Analgesia mediated by the TRPM8 cold receptor in neuropathic pain

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Declaration

I hereby declare that the composition of this thesis and the work presented are entirely my own. Some of the studies presented have been published as a research article. Reprints of published work are included in the appendix.

Clare Proudfoot MA (Hons)

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Abstract

Chronic pain, particularly neuropathic pain, is a major clinical problem which currently represents a largely unmet therapeutic need.

To identify novel analgesic strategies for chronic pain, we investigated the phenomenon of analgesia produced by cutaneous cooling. The recent identification of specific cold sensory receptors has allowed, for the first time, investigation of the molecular mechanism underlying cooling-induced analgesia.

We have shown that the cold-and-menthol receptor, TRPM8, is critically involved in cooling-induced analgesia. Activation of TRPM8 in a subpopulation of sensory afferents (by either cutaneous or intrathecal application of pharmacological agents or by modest cooling) elicits analgesia in neuropathic and other chronic pain models in rats, and inhibits the characteristic sensitisation of dorsal horn neurons that occurs ipsilateral to nerve injury. This analgesia is abolished following antisense knockdown of the TRPM8 receptor.

In contrast, activation of the related putative cold-receptor TRPA1 produces hyperalgesia in naïve and neuropathic rats.

TRPM8 expression was observed in small diameter sensory neurons in dorsal root ganglia and on afferent terminals in the spinal cord, with increases in specific subsets of sensory neurons following nerve injury.

We further found that the central mechanism of TRPM8-mediated analgesia is mediated through inhibitory Group II/III metabotropic glutamate receptors, and is opioid-independent.

These results identify TRPM8 as an essential molecular mediator of cooling-induced analgesia. We propose a novel analgesic axis in which activation of TRPM8-expressing afferents by innocuous cooling or chemical ligands leads to activation of inhibitory Group II/III metabotropic glutamate receptors in the spinal cord, which then exert inhibition over nociceptive inputs. These findings suggest that both TRPM8 and the inhibitory metabotropic glutamate receptors are promising targets for the development of novel analgesics for the treatment of neuropathic pain states.

List of Abbreviations

5-HT	5-hydroxytryptamine
AC	adenylyl cyclase
ACPC	1-aminocyclopropanecarboxylic acid
ACPT-III	(1R,3R,4S)-1-aminocyclopentane-1,3,4-tricarboxylic acid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid
AMPAR	AMPA receptor
ANOVA	Analysis of Variance
ATP	adenosine triphosphate
BCTC	(*N*-(4-tertiarybutylphenyl)-4-(3-chloropyridin-2-yl) tetrahydropyrazine-1(2*H*)-carboxamide
BLAST	Basic Local Alignment Search Tool
BSA	bovine serum albumin
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
cAMP	cyclic adenosine-monophosphate
Cat. No.	catalogue number
CCI	chronic constriction injury
CCK	cholecystokinin
cDNA	complementary DNA
CFA	Complete Freund's adjuvant
CGRP	calcitonin-gene-related-peptide
CHO	Chinese hamster ovary
CNS	central nervous system
con	contralateral
CREB	cAMP response element binding protein
DAG	diacylglycerol
DADS	diallyl disulphide

DC	direct current
DMEM	Dulbecco's modified Eagle's medium
DMF	dimethylformamide
DMSO	dimethylsulphoxide
DNA	dideoxyribonucleic acid
DNIC	diffuse noxious inhibitory control
DRG	dorsal root ganglia
EC_{50}	concentration required for 50% maximal effect
ED_{50}	dose required for 50 % maximal effect
ECL	Enhanced Chemiluminescent detection
EDTA	ethylenediaminetetraacetic acid
ENaC	epithelial Na^+ channel
EPSC	excitatory postsynaptic current
EPSP	excitatory postsynaptic potential
ERK	extracellular signal related kinase
fMRI	functional magnetic resonance imaging
g	grams
GABA	gamma-amino-butyric acid
GAPDH	glyceraldehyde -3-phosphate dehydrogenase
GDNF	glial-derived neurotrophic factor
GluR1-4	AMPA-receptor subunits GluR1- GluR4
GRIP	glutamate-Receptor-Interacting-Protein
HBSS	Hank's Buffered Saline Solution
HPETE	hydroperoxyeicosatetratenoic acid
HRP	horseradish peroxidase
HTcold	"High-threshold" cold sensitive afferents
i.p.	intraperitoneal
$iPLA_2$	Ca^{2+}-independent phospholipase A_2
i.t.	intrathecal
IASP	International Association for the Study of Pain
ipsi	ipsilateral

IP_3	inositol trisphosphate
IB-4	isolectin B-4
kDa	kiloDalton
l	litre
L3,4,5,6	lumbar level 3,4,5,6 (of the spinal cord)
LAP-4	L-(1)-2-amino-4-phophonobutyric acid
LI – X	lamina I – lamina X of the spinal cord
LTcold	"Low-threshold" cold sensitive afferents
LTP	long-term potentiation
m	metre
m	milli-
M	Molar
MAPK	mitogen activated protein kinase
mEPSC	miniature excitatory postsynaptic current
mg	milligrams
mGluR1-8	metabotropic glutamate receptor 1-8
min	minutes
mN	milliNewton
mol	moles
mRNA	messenger ribonucleic acid
ms^{-1}	metres per second
MΩ	megaohms
n	nano-
nA	nano-Amperes
NeuN	neuronal nuclei (marker)
NF-200	neurofilament-200 kDa
NGF	nerve growth factor

NMDA	N-methyl-D-aspartate
NMDAR	NMDA-receptor
NR1-3	NMDA-receptor subunits
NSAIDs	non-steroidal anti-inflammatory drugs
NSF	N-ethylmaleimide-sensitive fusion protein
°C	degrees centigrade
PAD	primary afferent depolarisation
PAG	periaqueductal grey matter
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PGE_2	prostaglandin E_2
PICK-1	protein-Interacting-with-C-Kinase-1
PIP_2	phosphatidylinositolbisphosphate
$PI(4,5)P_2$	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PNL	partial nerve ligation
PSD-95	postsynaptic density protein-95
PWL	paw withdrawal latency
PWT	paw withdrawal threshold
RACE-PCR	rapid amplification of cDNA ends-polymerase chain reaction
RM	repeated measures
rpm	revolutions per minute
RT-PCR	reverse transcriptase-polymerase chain reaction
RVM	rostral ventromedial medulla
SDS	sodium dodecyl sulphate

SEM	standard error of the mean
SG	substantia gelatinosa
SNI	spared nerve injury
SNL	spinal nerve ligation
SP	substance P
SP-SAP	substance P-saporin conjugate
SPET	suspended paw elevation time
TENS	transcutaneous electrical nerve stimulation
TM	transmembrane
TNF- α	tumour necrosis factor- α
Tris	tris-hydroxymethylaminoethane
TRP channel	Transient-receptor-potential channel
TRPA1	Transient-receptor-potential ankyrin 1 channel
TRPM8	Transient-receptor-potential melastatin 8 channel
TRPV1	Transient-receptor-potential vanilloid 1 channel
TRPV2	Transient-receptor-potential vanilloid 2 channel
TRPV3	Transient-receptor-potential vanilloid 3 channel
TRPV4	Transient-receptor-potential vanilloid 4 channel
TTX-R	tetrodotoxin-resistant
TTX-S	tetrodotoxin-sensitive
V	volts
w/v	weight divided by volume (expressed as percentage)
WDR	wide dynamic range (neuron)
μ	micro-

Chapter 1: Introduction

1.1 Physiological and chronic pain

Pain is a normal part of human experience. Physiological pain serves a useful purpose: alerting organisms to damaging stimuli and triggering withdrawal reflexes, or forcing a state of rest to promote recovery from injury. In contrast, chronic pain persists long after the original injury has healed, and therefore does not serve any such useful purpose. Although definitions (and thus reports) of chronic pain vary immensely, chronic pain is a major clinical problem: a recent epidemiological study of more than 46,000 people in 16 European countries found that nearly one in five adults were affected by chronic pain (www.painineurope.com). Common sources of chronic pain are neuropathic pain, cancer pain, arthritic pain, back pain and headache. Chronic pain has major implications for the social, economic, and mental health of sufferers - for instance on the ability to work, or incidence of depression, and therefore poses a major socio-economic burden (Nicholson & Verma, 2004). Chronic pain states involve changes in the nervous pathways that process pain signals, so that they become more excitable, which results behaviourally in symptoms of hyperalgesia (lowered thresholds to painful stimuli), allodynia (the perception of normally innocuous stimuli as painful) and spontaneous pain. In order to understand the changes in the nervous system that may underlie chronic pain states, a consideration of the mechanisms of normal pain processing is necessary.

1.2 Introduction to basic mechanisms of nociception

The term nociception refers to the detection of noxious stimuli, which may result in the perception of pain. Noxious stimuli to the periphery or the internal organs activate specialised sensory afferents, which synapse in the dorsal horn of the spinal cord. Spinal cord projection neurons in turn connect to various specific brain areas. This outline is discussed in detail below.

1.3 Somatosensory afferents

The axons of primary afferent neurons run from sensory endings in target tissues to central synaptic terminals in the central nervous system (CNS), and have their cell bodies in dorsal root ganglia (DRG) near the spine or in the cranial nerve ganglia in the head (Gardner et al., 2000). The peripheral terminals of the primary afferents constitute the receptive part of the neuron, and have contact with specific skin areas from which the afferent fibres can be excited (known as the receptive field). The peripheral receptive terminals of the afferents are specialised to respond to specific sensory stimuli such as temperature, mechanical pressure and chemical agents. Proprioceptive afferents are a specific class of somatosensory afferents that detect internal information about the position and movement of the body and limbs; this class will not be discussed further in this thesis. Peripheral sensory stimuli activate the receptive terminals of sensory neurons, producing a local depolarisation of the neuronal membrane, which, if it is of sufficient strength, will trigger the firing of action potentials (Gardner & Martin, 2000).

Sensory afferents can be classified experimentally by their conduction velocity, by their selective responses to sensory stimuli, and by differential expression of certain characteristic proteins.

1.3.1 Conduction velocity of sensory afferents

Afferent sensory neurons can be classified into 3 main types by fibre conduction velocity. Aα fibre neurons have the largest diameter myelinated axons, and the highest conduction velocity (>30 ms^{-1} in the rat), and are often grouped together with Aβ fibres, which also have large-diameter, myelinated axons and a high conduction velocity - 14-30 ms^{-1} in the rat. Aδ fibres have medium-diameter, thinly myelinated axons, and a conduction velocity of between 2.2-14 ms^{-1} in the rat. C fibres have small-diameter, unmyelinated axons, and conduct slowly - <2.2 ms^{-1} in the rat (Harper & Lawson, 1985).

1.3.2 Selective sensitivity of afferents to sensory stimuli

Different classes of sensory afferents respond selectively to specific sensory stimuli. Non-nociceptive mechanoreceptors respond to a variety of innocuous tactile sensations including light touch, pressure, vibration, stretch, and hair movement (Gardner et al., 2000; Lynn & Carpenter, 1982; Willis & Coggeshall, 1991). These innocuous mechanoreceptors are generally associated with Aα and Aβ fibres (Lynn & Carpenter, 1982). However, a population of Aδ fibre neurons is associated with low-threshold mechanosensitive D hair follicle receptors (Djouhri & Lawson, 2004; Lynn & Carpenter, 1982), constituting ~27% of Aδ afferents in the rat sural nerve (Leem et al., 1993). A significant population of low-threshold mechanosensitive C fibres also exists, which were found to constitute up to 15% and 33% respectively of the total C fibre population within rat plantar and sural nerves (Leem et al., 1993; Lynn & Carpenter, 1982).

Non-nociceptive thermoreceptors respond to innocuous warm temperatures and warming, or to innocuous cool temperatures and cooling (Iggo, 1959; Iggo, 1969) and are mainly associated with Aδ and C fibre neurons. Afferents responding to innocuous cool temperature information will be discussed in greater depth later in section 1.19.1

Nociceptors, as defined by Sherrington, are activated exclusively by noxious stimuli that may be expected to evoke pain (Sherrington, 1906). The majority of nociceptive neurons are generally accepted as being associated with Aδ or C fibre neurons (Gardner et al., 2000). However, recent evidence indicates that a substantial population (approximately 20% in rat) of Aα/β fibre neurons may also respond preferentially to noxious stimuli (Djouhri & Lawson, 2004; Fang et al., 2002; Fang et al., 2005; Lawson, 2002).

All Aδ nociceptors respond to high-threshold noxious mechanical stimuli, but a percentage (~25%) is also responsive to high-threshold heat stimuli (Fang et al., 2005; Leem et al., 1993; Raja et al., 1999). Varying percentages of Aδ nociceptors responding to noxious cold have been reported – these and noxious cold-sensitive C fibres will be discussed in greater depth in section 1.19.2.

Most C fibre nociceptors are polymodal, responding to noxious heat, chemical, and mechanical stimuli, and in some cases to intense cold (Bessou & Perl, 1969;

Fang et al., 2005; Leem et al., 1993; Raja et al., 1999). Noxious stimuli evoke both a rapid sharp component of pain, and a delayed, dull component; it is thought from psychophysical evidence and from differences in the conduction velocities that A fibre nociceptors mediate the former and C fibres the latter (Basbaum & Jessell, 2000). Some nociceptors are classified as "silent" or "sleeping" nociceptors, as they do not appear to be active under normal circumstances, becoming responsive only when sensitised by tissue injury (Handwerker et al., 1991; McMahon & Koltzenburg, 1990; Schmidt et al, 1995).

Most classical descriptions refer to cutaneous nociceptors; however, nociceptor properties vary significantly with tissue type. In certain tissues such as the cornea or teeth, pain is evoked far more easily by stimuli which would be innocuous elsewhere (Julius & Basbaum, 2001), whereas visceral pain is poorly localised and dull and has no separate fast and slow components (Gebhart, 1996). These differences must be taken into account when considering pain arising from such tissues.

1.3.3 Sensory afferents show selective expression of characteristic proteins

Sensory afferents show differential expression of certain characteristic proteins, which can be useful experimentally. Myelinated afferents can be specifically labelled by antibodies against higher molecular weight neurofilament proteins (150-200 kDa) (Lawson & Waddell, 1991), whereas C fibre neurons can be specifically labelled by antibodies against the intermediate filament protein, peripherin (Amaya et al, 2000). There are two main populations of C fibres: a population of peptidergic neurons which contain the peptides Substance P (SP) or calcitonin-gene-related-peptide (CGRP), and which express the high-affinity nerve growth factor (NGF) receptor trkA (Averill et al., 1995); and a non-peptidergic population which can be labelled selectively with the plant lectin isolectin B-4 (IB-4 - Snider & McMahon, 1998) and which expresses the Ret receptor glial-derived neurotrophic factor (GDNF - Bennett et al., 1998).

1.4 Molecular mechanisms of nociception

Recent years have seen great progress in elucidating the molecular basis of sensory transduction, with the cloning of receptor proteins which transduce specific sensory stimuli and are expressed by afferent neurons (Julius & Basbaum, 2001). The majority of these receptors are ionotropic cation channels, which upon activation allow cations into the afferent terminal, leading to depolarisation and potentially to the generation of action potentials. The classic example of such a receptor is the heat-and-capsaicin-sensitive receptor TRPV1 (transient-receptor-potential-vanilloid-1, earlier called VR1) (Caterina et al., 1997). When expressed in heterologous cells, TRPV1 is activated by noxious heat, with an activation threshold of approximately 44°C, and by capsaicin (Caterina et al., 1997). This activation threshold and the response to capsaicin match the properties of native noxious-heat-sensitive sensory cells (Cesare & McNaughton, 1996), suggesting that TRPV1 at least partially mediates the sensory response to noxious heat (Caterina et al., 1997; Cesare et al., 1999). Electrophysiological and behavioural studies of TRPV1-knockout mice demonstrate an almost completely abolished response to capsaicin (Caterina et al., 2000; Davis et al., 2000). However, the knockouts show normal behavioural responses to noxious heat (Caterina et al., 2000; Davis et al., 2000) and appear to have at least as many neurons sensitive to noxious heat as normal mice (Woodbury et al., 2004). Therefore, TRPV1 appears to be the key determinant of capsaicin sensitivity, but is probably not the sole mediator of noxious heat sensitivity (Caterina et al., 2000; Davis et al., 2000; Woodbury et al., 2004). However, TRPV1 does contribute significantly to inflammatory thermal hyperalgesia, as this is strongly reduced in the knockout mice (Caterina et al., 2000). TRPV1 is also sensitive to protons (Caterina et al., 1997; Tominaga et al., 1998) and a significantly reduced response to H^+ was observed in dissociated DRG neurons in TRPV1 knockout mice, suggesting that TRPV1 may also partially mediate the response of afferent neurons to acid (Leffler et al., 2006). The discovery of TRPV1 has resulted in much research on the role of this channel in pain and temperature sensation in vivo and in vitro, and has stimulated the discovery of more sensory TRP (transient-receptor-potential) channels.

Further heat-sensitive TRP channels are TRPV2, TRPV3 and TRPV4. TRPV2 is activated by temperatures of around 52°C, but not by capsaicin, and is expressed predominantly by Aδ fibre neurons (Caterina et al., 1999). Therefore TRPV2 may underlie the capsaicin-insensitive high-threshold heat response, which is characteristic of a class of Aδ nociceptors (Leem et al., 1993; Nagy & Rang, 1999). TRPV3 and TRPV4 are sensitive to innocuous warm temperatures and warming, with activation thresholds of ~35°C and ~34°C respectively (Peier et al., 2002a; Xu et al., 2002; Smith et al., 2002; Guler et al., 2002), and TRPV3 is also activated by the chemicals carvacrol, thymol, eugenol, camphor and menthol (Moqrich et al., 2005; Macpherson et al., 2006; Xu et al., 2006).

TRP receptors responsive to cold and to decreases in temperature form the basis of this thesis, and will be discussed in detail in section 1.24-1.25.

Additional receptors have been identified which are sensitive to peripheral stimuli such as acid (Leffler, 2006; Waldmann et al., 1999) and adenosine triphosphate (ATP) (Burnstock, 2005; Chen et al., 1995); a full discussion of peripheral sensory receptors is beyond the scope of this thesis. The identification of these specific receptor proteins has greatly advanced our understanding of the molecular mechanisms of pain sensation, although much further work remains to clarify the roles of individual receptors in in vivo responses to noxious stimuli.

1.5 Primary afferent termination in the spinal cord

The first synapse in the pain pathway is in the dorsal horn of the spinal cord, where primary afferent neurons terminate and form synapses with dorsal horn neurons (Basbaum & Jessell, 2000). Within the spinal cord grey matter, the pattern of afferent termination is highly ordered. The rostrocaudal and mediolateral organisation of the central terminals generate a somatotopic map of the body surface (Doubell & Mannion, 1999), at least in the superficial laminae (neurons in deeper laminae may not be somatotopically organised - Craig, 2003). Dorsoventrally, the spinal cord has a distinct cytoarchitecture, which was classified by Rexed (in the cat) into 10 laminae (Rexed, 1952). Figure 1.1 shows this laminar structure, adapted for the lumbar cord of the rat (Molander et al., 1984). Laminae I – VI (LI-LVI) comprise

the dorsal horn of the spinal cord. Different afferent types project to different laminae.

LI is the thinnest layer of the dorsal horn and receives input primarily from Aδ and C fibres (Willis & Coggeshall, 1991). Primary afferents terminating in LI generally form synapses directly with projection neurons, whose axons ascend to the brain (Giesler et al., 1979; Todd et al., 2002; Trevino & Carstens, 1975; Spike et al., 2003).

LII (also known as the substantia gelatinosa) receives input predominantly from C fibres, with some input from Aδ fibres (Basbaum & Jessell, 2000; Willis & Coggeshall, 1991). Afferents terminating in LII contact an interneuronal pathway, which relays the signal from afferents to projection neurons in LV (Braz et al., 2005; Snider & McMahon, 1998; Woolf & Fitzgerald, 1983).

LIII-IV receives input from A fibres, including both non-nociceptive Aδ fibres, originating from hair follicles, and large-diameter Aβ fibres originating from cutaneous mechanoreceptors (Brown & Iggo, 1967; Light & Perl, 1979). LIII-IV neurons also receive input from nociceptive fibres via excitatory interneurons (Willis & Coggeshall, 1991). The majority of afferents terminating in LIII and LIV form synapses directly with projection neurons that connect to the brain (Basbaum & Jessell, 2000; Giesler et al., 1979; Todd et al., 2002; Trevino & Carstens, 1975).

LV receives direct input from Aβ and Aδ fibres (Basbaum & Jessell, 2000; Basbaum & Fields, 1984; Craig, 2003; Willis & Coggeshall, 1991). LV neurons also receive input from C fibres, either onto their dendrites which extend dorsally into the superficial dorsal horn, or indirectly via excitatory interneurons (Basbaum & Jessell, 2000). Some LV neurons also receive nociceptive input from visceral structures (Basbaum & Jessell, 2000). Many LV neurons project directly to the brain (Basbaum & Jessell, 2000; Suzuki & Dickenson, 2005; Willis & Coggeshall, 1991).

Lamina VI only exists in the cervical and lumbosacral enlargements of the spinal cord where it represents the transitional layer between the dorsal and ventral horns. The main input to this lamina is from non-nociceptive afferents responding to joint manipulation (Basbaum & Jessell, 2000; Willis & Coggeshall, 1991).

The cranial afferent nerves terminate directly in the brain (in the brainstem) (Basbaum & Jessell, 2000). As this thesis has focussed on nerves in the body that terminate in the spinal cord, the cranial nerves will not be discussed further.

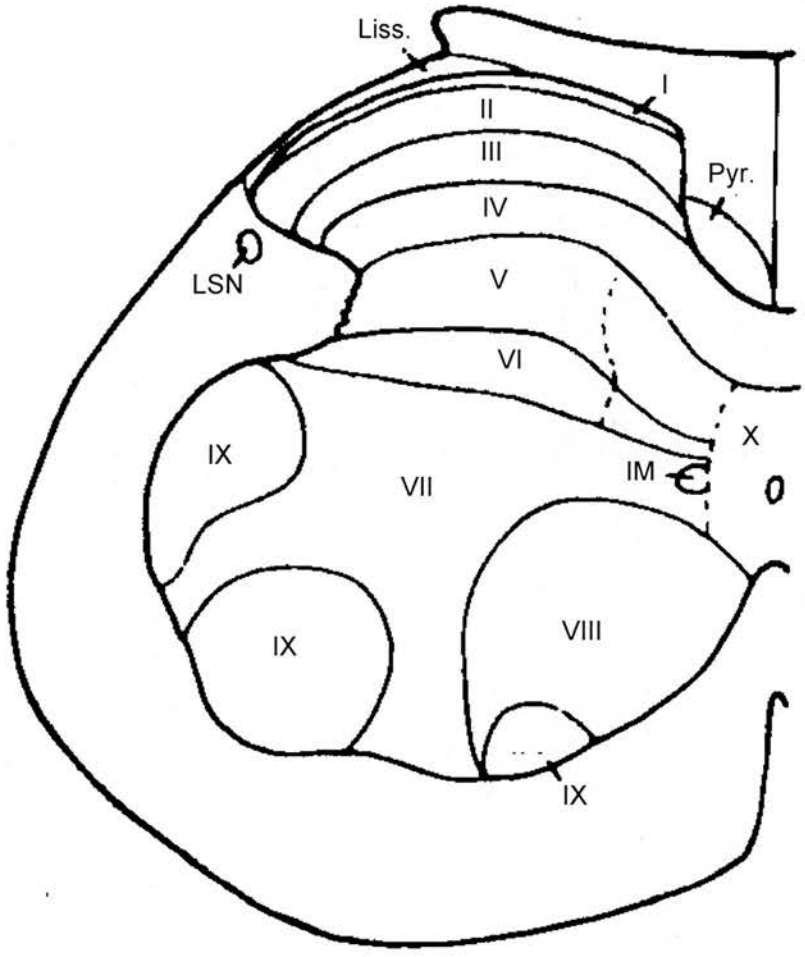


Figure 1.1 Laminae of the spinal cord
Figure adapted from Molander et al., 1984, shows the rat spinal cord at the rostrocaudal level L5, classified into laminae I-X. Laminae I-VI comprise the dorsal horn. "Liss." = Lissauer's tract "Pyr." = Pyramidal tract, "LSN" = lateral spinal nucleus, "VM" = ventromedial nucleus.

1.6 Responses of dorsal horn neurons to primary afferent input

Dorsal horn neurons may be classified into three basic types according to their response characteristics to primary afferent input. The first class responds exclusively to noxious information. These neurons receive inputs mainly from Aδ and C fibre neurons, and are located primarily in LI and LII (Christensen & Perl, 1970; Gregor & Zimmermann, 1972; Light & Perl, 1979). The second type of neurons respond only to innocuous information: these receive inputs from innocuous-mechanosensitive Aβ fibres, or from Aδ and C fibres signalling innocuous temperature information, and are located predominantly in LIII and LIV, and LI respectively (Craig, 2003; Willis & Coggeshall, 1991). The third class comprises multireceptive neurons, often known as "wide dynamic range" (WDR) neurons, which respond to both innocuous and noxious information. These neurons are most frequently found in the deeper dorsal horn laminae III-VI (Besson & Chaouch 1987; Willis & Coggeshall, 1991), but have also been identified in the superficial laminae (Iggo, 1974; Menétrey & Besson, 1981; McMahon & Wall, 1983; Woolf & Fitzgerald, 1983).

1.7 Dorsal horn neuronal output

Dorsal horn neurons are either long-distance excitatory projection neurons, whose axons ascend to the brain, or interneurons. The majority of dorsal horn neurons are interneurons (Willis & Coggeshall, 1991). Interneurons can be excitatory or inhibitory. They are activated by primary afferents, other interneurons, or descending fibres, and in turn, they synapse onto the presynaptic terminals of afferent neurons, onto the pre or postsynaptic terminals of other interneurons, or onto projection neurons (Willis & Coggeshall, 1991). Therefore, interneurons mediate a number of different roles within the spinal cord.

Neurons that project to the brain are found in LI and LIII-VI (Giesler et al., 1979; Spike et al., 2003; Todd et al., 2002; Trevino & Carstens, 1975), and these projection neurons connect to the thalamus, brainstem, midbrain, amygdala, hypothalamus, and striatum (Brooks & Tracey, 2005; Millan, 1999). The cerebral cortex is in turn

activated by projections from these areas. Processing of pain in the brain is outside the scope of this thesis and will not be discussed further.

1.8 Neurotransmission in the dorsal horn

There are extensive synaptic connections between primary afferents, interneurons, projection neurons, and descending fibres in the spinal cord, and a rich variety of neurotransmitters are involved in transmission at these different synapses. In general there exist multiple receptor subtypes for each neurotransmitter; therefore the effect of any transmitter is dependent on the particular receptor subtypes expressed at the synapse where the transmitter is released. The effect of neurotransmitters on dorsal horn processing is also determined by the type of neurons that they exert their effects at: for example, the activation of an inhibitory receptor located on an excitatory neuron will produce an inhibition of transmission, but the activation of the same inhibitory receptor on an inhibitory neuron will cause disinhibition, and thus increase dorsal horn excitability. Thus any one neurotransmitter can potentially exert a variety of effects, and this fact, coupled with the existence of multiple neurotransmitters, underlies the complexity of signal processing in the dorsal horn. Key neurotransmitters involved in dorsal horn signalling are discussed below.

1.8.1 Glutamate

The excitatory amino acid L-glutamate is the principal excitatory neurotransmitter of the nervous system (Kandel & Siegelbaum, 2000). In the spinal cord, glutamate is released by all primary afferent neurons, and is also released by excitatory interneurons (Willis & Coggeshall, 1991). Glutamate activates ionotropic receptors to directly open ion channels, and G-protein-coupled metabotropic receptors to cause longer-term modulatory changes. Ionotropic receptors comprise α-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid (AMPA) receptors, N-methyl-D-aspartate (NMDA) receptors, and kainate receptors (Hollman & Heinemann, 1994; Kandel & Siegelbaum, 2000). Metabotropic glutamate receptors (mGluRs) comprise mGluR1-8 and are subdivided into Groups I-III.

1.8.2 AMPA receptors

AMPA receptors (AMPARs) are predominantly postsynaptic in the dorsal horn (Coggeshall & Carlton, 1997; Henley et al., 1993), and mediate the majority of glutamate-induced postsynaptic excitatory current (Dougherty et al., 1992). AMPARs are composed of combinations of four possible subunits (GluR1-4), which form a tetrameric receptor (Hollmann & Heinemann, 1994). The GluR2 subunit undergoes post-transcriptional editing, which confers a low Ca^{2+}-permeability on the tetrameric receptor (Burnashev et al., 1992; Washburn et al., 1997), and thus the effect of AMPAR activation is a fast inward Na^{+} current, producing depolarisation and therefore possible action potential firing (Kandel & Siegelbaum, 2000). However, studies also indicate significant expression of Ca^{2+}-permeable AMPARs in dorsal horn neurons (Albuquerque et al., 1999; Tong & MacDermott, 2006), and activation of Ca^{2+}-permeable AMPARs can produce a transient change in synaptic strength at dorsal horn synapses (Engelman et al., 1999; Gu et al., 1996). Thus in addition to their primary role in postsynaptic excitation, AMPARs may also contribute to synaptic plasticity in the spinal cord.

1.8.3 NMDA receptors

NMDA receptors (NMDARs) are composed of NR1, NR2A-D and NR3A/B subunits, which form a tetrameric receptor, composed of two NR1 subunits together with two NR2 subunits, or with one NR2 and one NR3 subunit (Hollman & Heinemann, 1994). NMDARs are strongly expressed in the dorsal horn with NR1 and NR2B being particularly concentrated in the superficial dorsal horn, and are predominantly postsynaptically located (Coggeshall & Carlton, 1997; Tolle et al., 1993; Petralia et al., 1994).

Under normal circumstances, the NMDAR is blocked in a voltage-dependent manner by a Mg^{2+} ion in the channel pore, and requires simultaneous depolarisation-induced relief of this block and glutamate binding in order to open; therefore this receptor could be considered to function as a coincidence detector (Bliss & Collingridge, 1993). The NMDAR is also regulated by a glycine-binding site (Kleckner et al., 1988). Glycine appears to function as a co-agonist of the NMDAR, and antagonists of this site can attenuate the hyperalgesic effect of intrathecally

applied NMDA in vivo (Kolhekar et al.,1994), and the effect of NMDA on dorsal horn neuron responses (Dickenson & Aydar, 1991) highlighting the importance of interactive effects of neurotransmitters in the spinal cord.

The NMDAR is permeable to Ca^{2+}, and thus its activation leads to activation of Ca^{2+}-dependent intracellular signalling pathways, and resultant longer-term effects on neurons. The coincidence-detection properties and Ca^{2+}-permeability of the NMDAR allow it to play a crucial role in synaptic plasticity in the dorsal horn as in other nervous system areas (Bliss & Collingridge, 1983; Dickenson & Sullivan, 1987; Dougherty et al., 1992). The contribution of the NMDAR to spinal cord plasticity and the development of central sensitisation will be discussed below in section 1.15.2.

1.8.4 Kainate receptors

Kainate receptors are composed of the subunits GluR5, GluR6, GluR7, KA1 and KA2, forming a tetrameric receptor (Dingledine, 1999) that carries an excitatory current. Kainate receptors are strongly expressed in the superficial dorsal horn on postsynaptic terminals (Coggeshall & Carlton, 1997; Petralia et al., 1994) and are also found on presynaptic afferent terminals (Kerchner et al., 2001; Lucifora et al., 2006).

1.8.5 Group I metabotropic glutamate receptors

Group I mGluRs comprise mGluR1 and mGluR5. Immunoreactivity for mGluR1 and mGluR5 is concentrated in the superficial dorsal horn, and is seen at high levels on postsynaptic terminals of spinal cord neurons (Berthele et al., 1999, Jia et al., 1999). Group I mGluRs are also expressed in afferents, as immunoreactivity is observed in DRG (Hudson et al., 2002) and on peripheral terminals (Zhou et al., 2001). Group I mGluRs are coupled to phospholipase C (PLC)-mediated phosphoinositide hydrolysis and to formation of cyclic adenosine-monophosphate (cAMP) (Aramori & Nakanishi, 1992). Both of these effects contribute to cell excitability, and Group I mGluRs are therefore presumed to play a facilitatory role in synaptic transmission. In the spinal cord, Group I mGluRs are implicated in nociceptive behaviour and neuronal responses to noxious stimuli, as intrathecal

application of Group I mGluR agonists elicits nociceptive behaviours (Fisher et al., 1996), whereas antagonists inhibit dorsal horn neuronal responses to noxious stimuli (Young et al., 1994, 1995, 1997).

1.8.6 Inhibitory metabotropic glutamate receptors

Although most glutamate receptors are excitatory, the Group II/III mGluR subtypes exert inhibitory influences. Group II and Group III mGluR subtypes are a focus of this thesis, so will be covered in more detail than the other neurotransmitter receptors of the spinal cord.

1.8.7 Group II metabotropic glutamate receptors

Group II comprises mGluR2 and mGluR3. Activation of Group II mGluRs is coupled through the G_i/G_o protein to inhibition of cAMP formation (Bushell et al., 1999; Conn & Pin, 1997; Gereau & Conn, 1995). Activation of Group II mGluRs has been shown to inhibit N, L, and P/Q-type Ca^{2+} currents (McCool et al., 1996; Neugebauer, 2002; Robbe et al., 2002) which would cause functional inhibition of neurons.

Group II mGluRs are found in the spinal cord (Carlton et al., 2001; Jia et al., 1999; Petralia et al., 1996; Tang & Sim, 1999). Immunohistochemistry reveals that these receptors are located both presynaptically on the terminals of primary afferents in superficial laminae, with dorsal rhizotomy removing much, but not all, staining (Carlton et al., 2001); and on dorsal horn neurons and glia, particularly in deeper dorsal horn (Carlton et al., 2001; Jia et al., 1999; Tang & Sim, 1999). In afferent neurons, mGluR2/3 expression is particularly high among presumed nociceptive populations. Approximately 76% of mGluR2/3 immunoreactive DRG cells are IB-4 labelled, and in turn approximately 67% of IB-4-positive DRG cells are mGluR2/3-positive. Both myelinated (28%) and unmyelinated (32%) axons are mGluR2/3-positive (Carlton et al., 2001). In the dorsal horn, staining is particularly high in inner LII (Carlton et al., 2001; Jia et al., 1999), which is consistent with a high expression in IB-4-labelled afferents (Braz et al., 2005). Electron microscopy shows that Group II mGluR immunoreactivity in spinal cord neurons is found both at postsynaptic terminals and on the presynaptic terminals of both glutamatergic and GABAergic

interneurons (Jia et al., 1999; Neugebauer, 2002). mGluR2/3 immunoreactivity appears to be primarily located at extrasynaptic sites rather than at the synapse itself (Azkue et al., 2000), which is consistent with a potential heterosynaptic mechanism of activation. The localisation of the Group II mGluRs in the spinal cord is therefore consistent with a potentially complex role in the influence of nociceptive transmission, by presynaptic and/or postsynaptic mechanisms.

Group II mGluRs have been strongly implicated in an inhibitory role in spinal cord pain processing. Intrathecally applied specific agonists of Group II mGluRs attenuate pain behaviours in models of sensitised pain states: both in carrageenan and formalin-induced inflammatory pain (Dolan & Nolan, 2002; Simmons et al., 2002) and in the chronic constriction injury and spinal nerve ligation models of neuropathic pain (Fisher et al., 2002; Simmons et al., 2002). Electrophysiologically, Group II mGluR agonists reverse the sensitisation of spinal projection neurons produced by peripheral capsaicin or carrageenan-evoked inflammation, with little effect on normal transmission (Neugebauer et al., 2000; Stanfa & Dickenson, 1998). Group II mGluR agonists were also shown to inhibit A fibre-evoked potentials in dorsal horn neurons, an effect which appeared to be mediated presynaptically, as the frequency, but not the amplitude, of postsynaptic potentials was affected (Gerber et al., 2000). Therefore the evidence suggests that spinal Group II mGluRs play an inhibitory role in nociceptive transmission, which appears to be significantly upregulated in sensitised pain states.

1.8.8 Group III mGluRs

The Group III mGluRs comprise mGluR4, mGluR6, mGluR7 and mGluR8, of which mGluR4 and 7 have been shown in the spinal cord. Similarly to Group II, Group III mGluRs are linked through the G_i/G_o protein to inhibition of adenylyl cyclase (AC)-stimulated cAMP formation (Neugebauer, 2002; Okamoto et al., 1994). mGluR7 and mGluR4 activation has also been shown to inhibit N-type and P/Q type Ca^{2+} channels (Millan et al., 2003; Perroy et al., 2000). Therefore activation of Group III mGluRs has an inhibitory effect at the cellular level.

mGluR7 is highly expressed by DRG cells, and is found presynaptically on afferent terminals in the dorsal horn, with high levels in superficial laminae

(Kinoshita et al., 1998; Li et al., 1996; Li et al., 1997; Ohishi et al., 1995a,b). Dorsal rhizotomy removes most expression, indicating a location predominantly on afferent terminals (Ohishi et al., 1995b), although low levels of mRNA are found in the spinal cord, suggesting expression by spinal neurons also (Berthele et al., 1999; Boxall et al., 1998; Valerio et al., 1997). Electron microscopy shows an almost exclusive location in presynaptic active zones (Kinoshita et al., 1998), indicating that any expression of mGluR7 on spinal cord neurons is almost entirely due to expression on presynaptic terminals of spinal cord interneurons (Neugebauer, 2002). Of mGluR7-labelled primary afferents, roughly 33% and 10% also stain for IB-4 and SP respectively, and most IB-4-positive afferents, together with some SP-positive afferents, are labelled by mGluR7 (Li et al., 1997), which indicates a high degree of expression by nociceptive afferent populations, and is consistent with the high levels of mGluR7-immunoreactivity in dorsal horn superficial laminae. mGluR4 protein is also seen in small to medium DRG cells, and in the superficial dorsal horn, particularly LII, which is again consistent with expression by nociceptive primary afferents, and electron microscopy indicates that most mGluR4-immunoreactivity is in presynaptic terminals (Azkue et al., 2001). Low levels of mGluR4 mRNA are also found in the spinal cord (Berthele et al., 1999; Valerio et al., 1997), indicating possible expression by some spinal cord neurons. The location of both these receptors on the terminals of nociceptive primary afferents, and to a lesser extent on spinal cord neurons, is consistent with a role in modulating nociceptive transmission.

Spinal Group III mGluRs have been shown to inhibit transmission of nociceptive information. Intrathecally applied Group III mGluR agonists (which currently are unable to select between mGluR4/7) slightly decreased nociceptive responses in the formalin test (Fisher et al., 2002), and produced analgesia in a model of neuropathic pain (Chen & Pan, 2005). Ionophoresed agonists decreased the responses of spinal dorsal horn neurons to both innocuous and noxious mechanical stimuli, in both normal and capsaicin-sensitised animals (Neugebauer et al., 2000), suggesting that Group III mGluRs are less selective in their inhibitory effect than Group II mGluRs, which only affected sensitised responses. However, in a model of neuropathic pain, agonists inhibited the responses of sensitised ipsilateral dorsal horn neurons, but had no effect in naïve animals, and had similar effects behaviourally, suggesting that

Group III mGluR function may in fact be elevated in the neuropathic state (Chen & Pan, 2005). Similarly to the Group II mGluRs, agonists have also been shown to inhibit A fibre-evoked potentials in dorsal horn neurons, through an apparently presynaptic mechanism (Gerber et al., 2000). Overall, the evidence supports a role for the Group III mGluRs in the inhibition of spinal nociceptive transmission, although this may not be completely selective, as they have also been shown to have inhibitory effects on responses to innocuous stimuli (Neugebauer et al., 2000).

1.8.9 GABA and glycine

The amino acids gamma-amino-butyric acid (GABA) and glycine are inhibitory neurotransmitters that are released by populations of spinal inhibitory interneurons (Willis & Coggeshall, 1991).

Immunoreactivity for GABA is found in approximately 33% of LI-III spinal cord neurons (Hunt et al., 1981, Todd, 1996). Ionophoresis of GABA results in inhibition of dorsal horn neuron activity (Curtis et al., 1959, 1977; Zieglgansberger & Sutor, 1983), and intrathecal application of the GABA-A receptor antagonist bicuculline produces behavioural allodynia in rats, indicating that tonic inhibitory GABAergic mechanisms may control behavioural sensitivity (Yaksh, 1989). GABA receptors comprise ionotropic GABA-A receptors, which couple directly to Cl^- channels, and metabotropic GABA-B receptors, which are coupled to AC inhibition. Expression of both receptor types is observed in the spinal cord, with high levels in superficial dorsal horn, and is strongly decreased, but not abolished, by dorsal rhizotomy, indicating expression both on primary afferent terminals and on dorsal horn neurons (Alvarez et al., 1996; Coggeshall & Carlton, 1997; Price et al., 1987). Therefore GABAergic inhibition may operate by both pre and postsynaptic mechanisms.

Glycine is also implicated in inhibitory control of nociceptive transmission as intrathecal application of the glycine receptor antagonist strychnine produces behavioural allodynia in rats (Yaksh, 1989). Glycine receptor expression is observed throughout the dorsal horn, with the highest staining observed in deeper laminae (Coggeshall & Carlton, 1997; Todd & Spike, 1993); and is predominantly observed on postsynaptic membranes (Coggeshall & Carlton, 1997; Todd, 1996; van den Pol et al., 1988). Therefore glycine may produce inhibition by a mainly postsynaptic

mechanism, and may produce a stronger inhibitory effect on neurons in deeper laminae.

1.8.10 Neuropeptides

A variety of neuropeptides exert effects in the dorsal horn, including SP, CGRP, opioids, neuropeptide-Y, cholecystokinin (CCK), galanin, vasoactive-intestinal peptide, pituitary adenylyl cyclase-activating polypeptide, somatostatin, vasopressin, and others (Dickinson & Fleetwood-Walker, 1999; Wiesenfeld-Hallin et al., 2002; Willis & Coggeshall, 1991). Neuropeptides act on receptors coupled to second messenger systems to produce slower, longer-lasting effects than those exerted by conventional transmitters on ionotropic receptors, including changes in gene expression (Hokfelt et al., 2000). Because there is generally no specific reuptake mechanism as there is for conventional transmitters, neuropeptides can diffuse considerable distances from their release site and exert effects on more neurons (Basbaum & Jessell, 2000; Hokfelt et al., 2000). Neuropeptides contribute to the complexity of dorsal horn processing, as the same neuron may release conventional transmitters such as glutamate or GABA together with several different neuropeptides (Hokfelt et al., 2000; Ju et al., 1987) and may thereby exert a variety of potential effects on target neurons. Neuropeptides exert significant effects on dorsal horn pain processing but are not a focus of this thesis and therefore will not be covered in further detail, apart from opioids, which are important in endogenous analgesic mechanisms and are discussed below.

1.8.11 Opioids

The importance of the opioidergic system in pain processing is underlined by the pre-eminent role of the opiate drug morphine in pain treatment. Opioids exert effects at peripheral, spinal and supraspinal levels (Basbaum & Fields, 1984). In the spinal cord, intrathecal administration of morphine produces behavioural analgesia (Yaksh & Reddy, 1981), and ionophoresis of opioids produces a strong inhibition of dorsal horn neuron responses to noxious stimulation (Fleetwood-Walker et al., 1988a). The main endogenous opioid peptide subtypes are the enkephalins, the dynorphins, β-endorphin, and endomorphins 1 and 2 (Basbaum & Fields, 1984; Millan, 1999;

Zadina et al., 1997), all of which are found in the dorsal horn (Cruz & Basbaum, 1985; Basbaum & Fields, 1984; Dun et al., 2000; Glazer & Basbaum, 1981; Gutstein et al., 1992; Martin-Schild et al., 1999, 1998; Tsou et al., 1986).

Opioid receptors form three classes: μ, δ and κ opioid-receptors, all of which are coupled to AC inhibition, activation of hyperpolarising K^+ currents and inhibition of Ca^{2+} currents (Connor & Christie, 1999; Millan, 2002), thus causing inhibition of transmitter release from primary afferents and hyperpolarisation of excitatory postsynaptic neurons (Dickenson & Suzuki, 2005). Enkephalins act as agonists of the μ and δ-opioid receptors, β-endorphin and endomorphins 1 and 2 are selective agonists of the μ-opioid receptor, and dynorphin acts preferentially at the κ-opioid receptor (Millan, 2002; Zadina et al., 1997). All three classes are expressed in the dorsal horn, with high levels of expression in superficial laminae, which is where most nociceptive afferents terminate, and are thus well-placed to influence nociceptive transmission (Arvidsson et al., 1995; Coggeshall & Carlton, 1997; Elde et al., 1995; Mansour et al., 1996).

1.9 Modulation of spinal cord excitability and the gate control theory

The first theory proposing modulation of nociceptive transmission in the spinal cord was the gate control theory of Melzack & Wall (Dickenson, 2002; Melzack & Wall, 1965, 1982). In this theory, which is shown as modified by Melzack & Wall, 1982, in Figure 1.2, both large Aβ fibres and small C/Aδ fibres activate dorsal horn transmission neurons and therefore the output from transmission neurons depends on the nature of the peripheral stimulus. However, Aβ fibres also activate inhibitory neurons in the substantia gelatinosa (SG; LII) which function as a "gate" by presynaptically inhibiting afferent activation of transmission neurons. The activation of Aβ fibres by innocuous tactile stimulation activates inhibitory neurons, partially closing the presynaptic gate, and thereby inhibiting transmission neuron firing. For pain to be produced it is necessary for the noxious stimulus strength to overcome this inhibition, and this in turn can be overcome by further stimulation of Aβ fibres, by an increase in the presynaptic inhibition of small cell firing. This suggests how rubbing

or other innocuous tactile stimulation of an injured area, or the use of transcutaneous electrical nerve stimulation (TENS) can relieve pain.

The gate control theory proposes that transmission through the dorsal horn can be modulated by activity in other classes of afferents. Such modulation has been demonstrated experimentally, for example the phenomenon of primary afferent depolarisation (PAD) (Rudomin & Schmidt, 1999; Willis, 2006). PAD is elicited in afferents by activity in neighbouring groups of afferents, and results in an inhibition of neurotransmitter release from the depolarised terminals (Eccles, 1961; Frank & Fuortes, 1957; Rudomin & Schmidt, 1999; Willis, 2006), demonstrating the existence of inhibitory effects produced by one class of afferents on another. In addition to proposing modulation of transmission by afferent input, Melzack and Wall emphasised the importance of descending control from the brain affecting dorsal horn neuron responses. While the experimentally known properties of the spinal cord do not precisely match with the gate control theory, the central tenets of modulation of dorsal horn nociceptive processing by activity in other classes of afferents and by descending controls from the brain remain fundamental to our working hypothesis of pain processing (Basbaum & Fields, 1984; Willis, 2006).

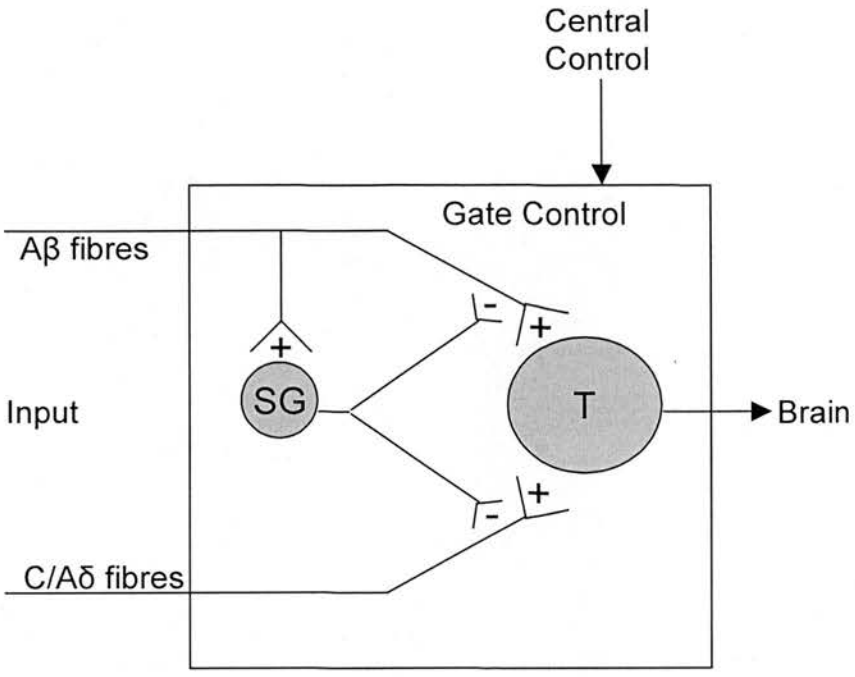


Figure 1.2 The Gate Control Theory
Illustration of the Gate Control Theory, adapted from Melzack & Wall., 1982. Both large Aβ fibres and small C/Aδ fibres activate dorsal horn transmission (T) cells; therefore the output from transmission cells depends on input stimulus. Aβ fibres also activate inhibitory cells in the substantia gelatinosa – SG. These function as a "gate" by inhibiting T cells presynaptically. Activation of Aβ fibres by innocuous tactile stimulation activates SG cells, closing the presynaptic gate, and thereby inhibiting transmission cell firing. For pain to be produced it is necessary for the noxious stimulus strength to overcome this inhibition.

1.10 Descending control of spinal excitability

Stimulation of brain areas such as the rostroventromedial medulla (RVM) (which includes the nucleus raphe magnus) and the periaqueductal grey matter (PAG), or microinjection of opiates into these areas, produces analgesia (Basbaum et al., 1976; Basbaum & Fields, 1984; Fields, 1977; Murfin et al., 1976; Willis et al., 1977). This analgesia can be blocked by lesion of the dorsolateral funiculus tract which descends to the dorsal horn, indicating that this mechanism operates via a descending pathway to the spinal cord (Basbaum & Fields, 1984). This descending pathway represents a major endogenous analgesic system, in which activation of an opioid-activated pathway from the PAG to the RVM, or direct activation of RVM neurons, results in activation of descending serotonergic and noradrenergic pathways from the RVM to exert inhibitory effects in the spinal cord (Basbaum & Fields, 1984; Fields 2004; Millan, 2002). RVM neurons synapse onto dorsal horn projection neurons, producing a direct inhibition via release of their transmitter: noradrenaline or serotonin (also known as 5-hydroxytryptamine - 5-HT) (Fields, 1999; Giesler et al., 1981). RVM neurons also synapse onto interneurons and may inhibit excitatory interneurons, and activate GABAergic and opioidergic inhibitory interneurons (Fields, 1999; Sorkin et al., 1993). Opioidergic interneurons of the spinal cord play a key role in this descending inhibition, as demonstrated by the fact that intrathecal naloxone can block the inhibition caused by stimulation of the RVM or by supraspinal opioid administration (Fields, 1999; Levine et al., 1982; Zorman et al., 1982). A number of different 5-HT receptor families are found in the dorsal horn: 5-HT_1, 5-HT_2, 5-HT_3 and 5-HT_7 (Coggeshall & Carlton, 1997; Suzuki & Dickenson, 2005) and of these the 5-HT_1 receptor family appears to play a significant role in the analgesic effects of descending RVM neurons, as ionophoresis of 5-HT_1 antagonists blocks the inhibition of dorsal horn neurons elicited by the electrical stimulation of descending fibres from the RVM (El-Yassir & Fleetwood-Walker, 1990).

Other brain areas are also implicated in descending inhibitory controls. Noradrenaline is released from fibres descending in the dorsolateral funiculus from the locus coeruleus, subcoeruleus, and A5 nucleus, in addition to the RVM (North & Yoshimura, 1984; Westlund et al., 1983; Yoshimura & Furue, 2006). The overall

effect of adrenergic transmission in the dorsal horn appears to be antinociceptive (Yoshimura & Furue, 2006), as intrathecal administration of adrenaline or the α_2 agonist clonidine is antinociceptive (Reddy & Yaksh, 1980), and α_2 agonists are used clinically as analgesics (Racle et al., 1987). Fibres releasing the monoamine dopamine descend to the spinal cord from brain areas including the A11 nucleus of the diencephalon, and stimulation of this area produces an inhibition of spinal neuron responses to noxious stimuli (Fleetwood-Walker et al., 1988b), as does spinal application of dopamine (Gao et al., 2001; Tamae et al., 2005) demonstrating an antinociceptive role of this transmitter.

In addition to descending inhibitory control, descending input can also be facilitatory. Activation of certain cells in the RVM (termed "on cells") can produce a facilitation of dorsal horn neuronal responses to noxious stimuli (Fields, 2004; Neubert et al., 2004; Suzuki et al., 2004; Zhuo & Gebhart, 1992). This facilitation may also be mediated through release of 5-HT from descending fibres, as 5-HT can produce facilitatory effects by its actions at excitatory receptor subtypes (Suzuki et al., 2002, 2004). For example, the 5-HT_3 receptor (which is a ligand-gated ion channel, whereas the other 5-HT receptor families act through G-proteins; Maricq et al., 1991) is implicated in a pronociceptive role in the dorsal horn, as antagonists of this receptor reduce behavioural and neuronal responses to nociceptive stimuli (Ali et al., 1996; Green et al., 2000). A full discussion of descending facilitatory input is outside the scope of this thesis, however, the existence of both facilitatory and inhibitory descending systems increases the range of modulation which can be produced by descending input, contributing to the complexity of pain processing.

1.11 Development of sensitised pain states

Following injury, sensitisation develops in the nervous system pathways that signal pain, and is manifest behaviourally as hyperalgesia, allodynia, and spontaneous pain. Both peripheral and central mechanisms are normally involved in the development of sensitisation. Inflammatory mediators, such as bradykinin and prostaglandins, produce sensitisation of the peripheral terminals of nociceptive neurons by modifying receptors and ion channels to result in increased sensitivity of

the nociceptor terminal to activating stimuli (Chuang et al., 2001; Julius & Basbaum, 2001). Centrally, there is a facilitation of dorsal horn neuron responsiveness to afferent input (which will be covered in section 1.15), which results in increased transmission from the dorsal horn to brain areas, where the pain signal is ultimately perceived. The phenomenon of sensitisation is a normal part of pain processing following tissue injury or inflammation, and is of benefit to an animal, promoting rest and guarding of an injury. As the injury recovers, the sensitisation resolves. However, in chronic pain states, this sensitisation becomes pathological, as it does not recover in parallel with recovery of the injury. Such altered pain states require long-lasting changes in the nervous system. One form of chronic pain is peripheral neuropathic pain, which is the principal type of chronic pain investigated in this thesis.

1.12 Neuropathic pain

Neuropathic pain is defined by the International Association for the Study of Pain (IASP) as pain initiated or caused by a primary lesion or dysfunction in the nervous system. Peripheral neuropathic pain arises from damage to the peripheral nerves whereas central neuropathic pain arises from damage in the CNS; the focus of this thesis is peripheral neuropathic pain. Peripheral neuropathic pain has a variety of causes including: physical damage to peripheral nerves such as compression, cut, stretch or various other injuries, as a result of accidental injury or surgical trauma, (Bridges et al., 2001; Macrae & Davies, 1999; Morley-Forster et al., 2006); demyelination of afferents as occurs in Charcot-Marie-Tooth and Guillain-Barré syndromes (Asbury, 1990; Carter et al., 1998); ischaemic injury caused by diabetes or alcoholism (Morley-Forster, 2006); damage to nerves by viral infection as occurs with HIV infection (Verma et al., 2005) and postherpetic neuralgia (Schmader et al., 2002); compression or invasion of neural structures by tumours, frequently within the bones (Coleman, 1997; Luger et al., 2005; Peters et al., 2005); and use of certain neurotoxic chemotherapy agents (Quasthoff & Hartung, 2002). Peripheral neuropathic pain affects 2.4% of the UK population, rising to 8% with age (Neuropathy Trust UK; www.neuropathytrust.org). Currently, neuropathic pain is

inadequately treated (Morley-Forster, 2006; Woolf & Mannion, 1999). Opioid drugs are less effective in neuropathic pain states than in other pain states, and require much higher doses to produce an analgesic effect (Moulin, 2006), which induce adverse side effects. The most effective medications in current use are anti-convulsant drugs such as gabapentin, and tricyclic antidepressants such as amitriptyline. All of these show variable efficacy, achieving clinically significant effects in less than 50% of patients (Bridges et al., 2001; Sindrup & Jensen, 1999), and may cause deleterious side effects. There is therefore a great need to develop new analgesic therapies for use in neuropathic pain conditions. Much of what is known about the mechanisms underlying neuropathic pain has been learned from animal models, which are discussed below.

1.13 Animal models of neuropathic pain

A number of experimental animal models have been developed to model pain resulting from peripheral nerve damage. One of the first models of peripheral nerve injury was that of complete sciatic nerve section or axotomy, which results in biochemical and functional changes within the nerve and the CNS (Wall et al., 1979). However, a key disadvantage of this model is that the whole sciatic territory is completely denervated, and this major sensory loss means that it is very difficult to perform behavioural measurements on this model. Therefore, this model has been largely abandoned in favour of partial nerve injury models, in which behavioural tests can be performed on the affected ipsilateral hindlimb, to measure the reflex sensitivity to mechanical and thermal stimuli. Additional models have also been developed to mimic specific neuropathic conditions, such as a varicella zoster virus model of postherpetic neuralgia (Fleetwood-Walker et al., 1999), a streptozotocin-induced model of diabetic neuropathy (Malcangio & Thompson, 1998), and a model of demyelination-induced pain (Wallace et al., 2003). The model of demyelination-induced pain was used in this thesis and is described below with the surgical models of neuropathic pain.

1.13.1 Chronic constriction injury

The chronic constriction injury (CCI) model (Bennett & Xie, 1988), which was used in experiments in this thesis, is produced by loosely tying four chromic cat gut ligatures around the common sciatic nerve, at mid-thigh level. This results in the development of lowered behavioural reflex thresholds to thermal and mechanical stimuli, and de novo withdrawal reflexes to 4°C cold, ipsilateral to injury, that are most manifest between 1 to 2 weeks following surgery (Bennett & Xie, 1988; Kim et al., 1997). Guarding behaviour of the injured limb is also observed (Bennett & Xie, 1998; Kim et al., 1997), which may indicate spontaneous pain.

Following the set-up of the injury, a neuroma forms at the site of injury, and swelling of the nerve is observed, resulting in compression and consequent axotomy of some of the axons (Bennett & Xie, 1988; Coggeshall et al., 1993; Maves et al., 1993). From day 3 to 2 weeks post-operatively there is an extensive loss of axons distal to the injury, with a particularly marked loss of large myelinated Aβ fibres (Basbaum et al., 1991; Coggeshall et al., 1993). An immune response to the chromic sutures may at least partially underlie the changes observed, as chromic gut but not plain or silk gut sutures produce altered paw position and thermal hyperalgesia (Maves et al., 1993).

This model was used in the following experiments. It was found to be easy to set up, and reliably generated behavioural sensitisation. Animals were not used (and hence the surgery deemed to have failed) if the sensitised limb showed a difference in latency of withdrawal to a thermal stimulus of less than 4.5 s, and a difference in mechanical threshold of less than 1500 mN/mm^2. Based on these criteria the failure rate was approximately 1 in 50. Furthermore, very low rates of surgical complications were observed, with less than 1 animal in 100 developing any problems associated with the surgery.

1.13.2 Partial nerve ligation

The partial nerve ligation model (PNL) involves a tight ligation of approximately one third to one half of the sciatic nerve (Seltzer et al., 1990). This model produces lowered behavioural reflex thresholds to thermal and mechanical stimuli and

development of cold allodynia in the ipsilateral hindlimb (Kim et al., 1997; Seltzer et al., 1990).

1.13.3 Spinal nerve ligation

Spinal nerve ligation (SNL) involves the tight ligation of the L5 and L6 spinal nerves, which contribute to the sciatic nerve, or alternatively ligation of the L5 nerve only, as this produces much the same effect (Kim & Chung, 1992; Kim et al., 1997). The nerve is ligated just distal to the DRG, thereby transecting all axons within the L5 branch. This model develops lowered behavioural thresholds to thermal and mechanical stimuli and withdrawal reflexes to 4°C cold ipsilateral to injury (Katsura et al., 2006; Kim & Chung, 1992). A key benefit of this model is the fact that the surgery is standardised, with the same group of fibres affected every time. This model also allows easy comparison of changes in damaged and undamaged afferents by comparison of the damaged L5 DRG with the spared L4 DRG (Bridges et al., 2001; Campbell & Meyer, 2006). One disadvantage of this model is that it is comparatively difficult surgery to perform, leading to raised rates of complications.

1.13.4 Spared nerve injury

In the spared nerve injury (SNI) model, the tibial and peroneal branches of the sciatic nerve are severed, leaving the sural intact (Decosterd & Woolf, 2000). Mechanical hypersensitivity and cold allodynia develop in the area innervated by the spared sural nerve branch. However, in contrast to other models, there is no decrease in latency to a noxious heat stimulus, although there is an increase in the duration of withdrawal to the stimulus, which perhaps demonstrates an increased noxiousness of the stimulus (Decosterd & Woolf, 2000). The key difference between this and the other models is that the damaged (severed) axons are separate from the spared axons, as they form separate branches of the nerve, distal to the injury. Despite this separation, hypersensitivity develops both in the area innervated by the spared sural branch of the nerve and, to a lesser extent, in areas innervated by the saphenous nerve, which is completely separate to the sciatic innervated area (Decosterd & Woolf, 2000). This indicates that there must be interactions between the damaged and undamaged afferents elsewhere, which could occur via mingling of intact and

injured peripheral terminals in the skin, as the sural and saphenous nerve territories lie immediately next to and overlap to an extent with the tibial and peroneal territories (Swett & Woolf, 1985), or alternatively could occur via mingling of injured and uninjured cell bodies in the same DRG (Decosterd & Woolf, 2000).

1.13.5 Lysolecithin model of demyelination-induced pain

Peripheral demyelinating diseases are associated with pain (Asbury et al., 1990, Carter et al., 1998). The lysolecithin model was set up to model the pain associated with demyelination. Focal application of the myelin toxin lysolecithin to the sciatic nerve produces demyelination of afferents with no axonal loss (Wallace et al., 2003). Behavioural thermal hyperalgesia and mechanical and cold allodynia result ipsilateral to the injury (Wallace et al., 2003; Results section 4.1). Spontaneous discharge in afferents is observed, and immunohistochemical analysis of the DRG shows some changes similar to those observed in surgical neuropathic models, including a decrease in expression of Nav1.8, and induction of Nav1.3 expression, and induction of NPY expression (Wallace et al., 2003), suggesting some similarities between demyelination-induced pain and neuropathic pain. However, no changes in expression of the peptides galanin and CGRP were observed, suggesting some important differences between this model and surgical models (Wallace et al., 2003).

A summary of key changes which have been observed in the surgical animal models of neuropathy and clinical cases are summarised in Figure 1.3, and include both peripheral changes in primary afferents, and central changes in the dorsal horn.

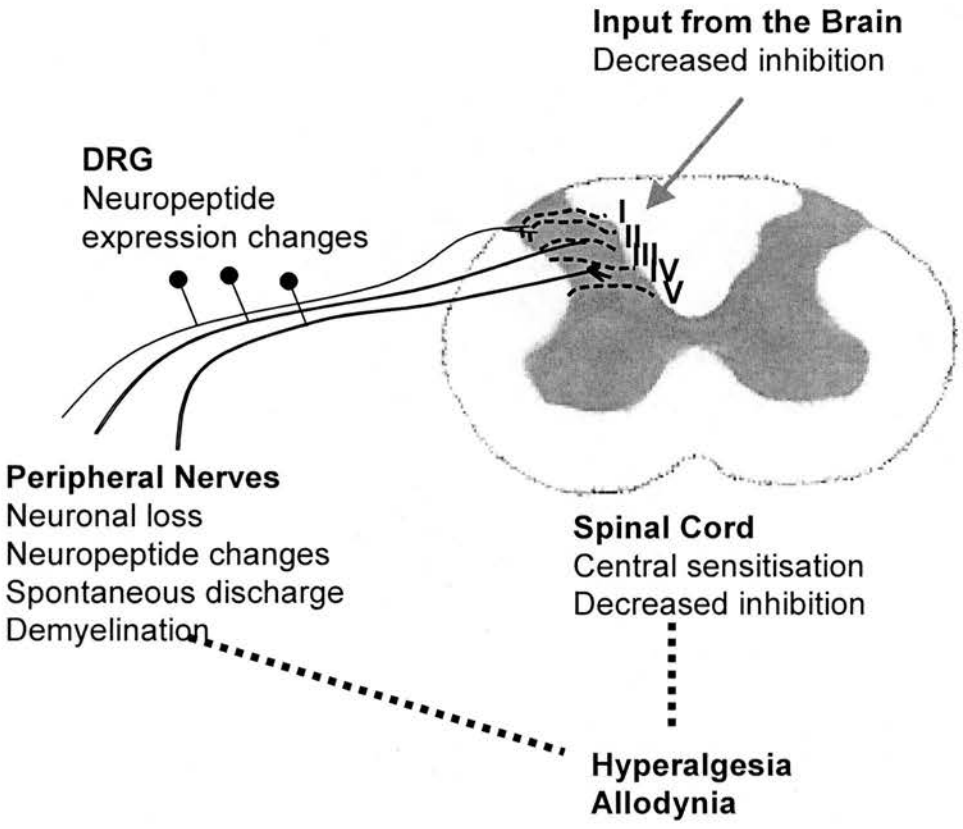


Figure 1.3 Alterations in pain processing pathways in neuropathic pain

Figure 1.3 summarises the alterations that occur following nerve damage which may underlie neuropathic pain. Peripheral nerves show axonal loss, altered firing properties, demyelination and changes in expression of neuropeptides and of receptor proteins. In the spinal cord, central sensitisation, and a loss of inhibitory tone occurs. Alterations also occur in descending input from the brain. All these effectively make the spinal cord more excitable and lead to behavioural hyperalgesia and allodynia.

1.14 Alterations in primary afferent neurons following peripheral neuropathy

The sensitisation of peripheral neuron terminals by inflammatory mediators is thought to play a much lesser role in neuropathic pain, as neuropathic pain can arise without significant tissue damage (Bridges et al., 2001; Treede, 1992). However, neuropathic pain models are associated with significant, neuropathy-specific changes in primary afferents, including alterations in electrical properties and in the expression of key proteins, and these changes are thought to contribute significantly to the generation of neuropathic sensitisation.

1.14.1 Alterations in afferent firing properties and ion channel expression following peripheral neuropathy

Following peripheral nerve injury, there is an increase in the level of spontaneous firing in afferent neurons within the nerve (Bridges et al., 2001). This firing can be described as ectopic, as it originates from areas other than the peripheral sensory terminal of the neuron. Such firing was first described in recordings from the neuroma (the area of nerve damage) (Wall & Gutnick, 1974). However, further studies demonstrated that spontaneous firing could also originate from the DRG and other points along the nerves (Wall & Devor, 1983), indicating that abnormal firing properties are present throughout the neurons, rather than localised at the neuroma. Spontaneous firing has been recorded in both damaged afferents and in their undamaged neighbours - for example, following SNL, spontaneous firing has been recorded in both neurons of the L5 (damaged) DRG (Liu et al., 2000; Ma et al., 2003; Wu et al., 2001) and in neurons of the spared L4 DRG (Ali et al., 1999; Djouhri et al., 2006; Liu et al., 2000; Ma et al., 2003). Microneurography in human patients with peripheral neuropathy has documented spontaneous firing in afferents (Calvin et al., 1982; Campero et al., 1998; Ochoa & Torebjork, 1980; Torebjork et al., 1979), supporting a role for such abnormal electrical phenomena in clinical cases of neuropathic pain.

The firing properties of afferent neurons are dependent on their expression of a repertoire of voltage-gated ion channels, and alterations in the expression of such channels has been observed in models of neuropathy, which may contribute to the

altered firing properties. Voltage-gated Na^+ channels mediate the majority of the action potential and therefore alterations in expression of these channels would be expected to have significant consequences on afferent firing properties. Nociceptive afferents express both tetrodotoxin-resistant (TTX-R) and tetrodotoxin-sensitive (TTX-S) Na^+ channel subtypes. Following CCI, there is a decrease in the TTX-R Na^+ current in DRG neurons, and in transcripts of the TTX-R channels $Na_v1.9$ and $Na_v1.8$ in the DRG (to levels of approximately 60% of normal levels; Dib-Hajj et al., 1999). Conversely, there is an increase in TTX-S Na^+ current in small-diameter DRG neurons, and de novo transcription of the TTX-S $Na_v1.3$ Na^+ channel, which is normally only found in DRG during development (from 0 to ~ 15 % DRG cells showing transcripts; Dib-Hajj et al., 1999; Wood et al., 2004). These changes may provide at least a partial basis for the hyperexcitablity of DRG neurons observed following CCI and axotomy. For example, the upregulation of the $Na_v1.3$ channel, which recovers rapidly from inactivation, could allow the neurons expressing this channel to fire repetitively at abnormally high frequencies (Cummins & Waxman, 1997). The importance of Na^+ channels in neuropathic pain is further suggested by the clinical usefulness of drugs that act on voltage-gated Na^+ channels, such as the anticonvulsant carbamazepine (Bridges et al., 2001; Rizzo, 1997).

1.14.2 Changes in neuropeptide expression following peripheral neuropathy

Changes are observed in the expression of neuropeptide transmitters and of their receptors in the dorsal horn following peripheral nerve injury (Hokfelt et al., 1994; Wiesenfeld-Hallin et al., 1999). For example, a decrease in the expression of SP (Kajander & Xu, 1995; Nahin et al., 1994) and CGRP (Nahin et al., 1994; Noguchi et al., 1993) is observed following peripheral nerve injury, whereas levels of galanin increase (Ma & Bisby, 1997; Nahin et al., 1994; Shi et al., 1999). Such alterations in expression may be accompanied by functional alterations – for example intrathecal administration of galanin attenuates sensitisation in CCI but not in normal rats (Liu & Hokfelt, 2000), suggesting an increased inhibitory role of this peptide following peripheral nerve injury. Neuropeptides exert significant long-lasting neuromodulatory changes in the dorsal horn and therefore alterations in neuropeptide expression following neuropathy may significantly influence spinal cord processing.

1.14.3 Changes in expression of peripheral receptors following peripheral neuropathy

Receptor proteins that transduce sensory stimuli in the periphery also show altered expression in neuropathic pain models. Such changes may be expected to affect the responsiveness of afferents to peripheral noxious stimuli, and thus could contribute to the abnormal pain behaviours observed following peripheral nerve damage.

An example of such a receptor that shows altered expression following nerve injury is the heat-and-capsaicin receptor TRPV1. In the SNL model, TRPV1-immunoreactivity decreases in L5 DRG, but increases in L4 DRG, suggesting that TRPV1 expression is lost from damaged afferents but is upregulated in spared neighbouring afferents (Fukuoka et al., 2002; Hudson et al., 2001). The situation following CCI is less clear: Western blots of spinal cord tissue showed an increase in TRPV1 protein ipsilateral to injury following CCI (Kanai et al., 2005), but immunohistochemical analysis of DRG tissue showed a decrease in TRPV1 levels (Schafers et al., 2003). If, as the SNL results suggest, TRPV1 expression is increased in undamaged afferents and decreased in damaged afferents, the discrepancies in the CCI model could be due to the mixing of damaged and undamaged afferents in the same DRG, which would lead to a much less clear picture of TRPV1 expression changes than the SNL model. Alterations in the expression of TRPV1 would be expected to affect the sensitivity of the affected area to noxious heat, and could thereby contribute to the abnormal processing of noxious heat information that is observed in neuropathic pain models. Similar changes in the expression of other receptors could contribute to the altered sensory processing observed in neuropathy.

1.14.4 Relative contributions of injured and neighbouring afferents to neuropathic sensitisation

Following partial nerve injury, the nerve contains both damaged and neighbouring undamaged afferents, both of which groups show altered firing properties (Liu et al., 2000; Ma et al., 2003) and altered protein expression (Hudson et al., 2001, 2002), and both of which may have a role to play in behavioural sensitisation.

Support for the role of injured afferents comes from data showing that, in the SNL model, low doses of local anaesthetics (Sukhotinsky et al., 2004) or tetrodotoxin

(Lyu et al., 2000) directed at the L5 DRG reduce behavioural hypersensitivitiy, suggesting that the activity of injured afferents is necessary for ongoing behavioural sensitisation. Some groups (Sheen & Chung, 1993; Sukhotinsky et al., 2004; Yoon et al., 1996) have found that cutting the L5/L6 dorsal roots abolishes SNL-induced behavioural sensitisation (Gold, 2000). However, other groups have not observed such a reversal of SNL-induced neuropathic sensitisation following L5 dorsal rhizotomy (Eschenfelder et al., 2000; Li et al., 2000), and neuropathic pain behaviour was produced following an L5 ganglionectomy, in which afferent input is removed altogether (Sheth et al., 2002), suggesting that the input from damaged afferents may not in fact be critical for hypersensitivity. In the SNI model, hypersensitivity is observed in areas innervated by the undamaged sural branch of the sciatic nerve (Decosterd & Woolf, 2002), suggesting that the undamaged afferents contribute to neuropathic hypersensitivity. Further support for this role of undamaged afferents comes from data showing that selective abolition of the input from undamaged afferents, by L4 rhizotomy, abolished pain behaviours evoked by L5 SNL (Li et al., 2000; Yoon et al., 1996).

Overall the evidence suggests a role for both injured and uninjured afferents in the abnormal processing of pain signalling following nerve injury.

1.14.5 Role of neurotrophic factors

Following peripheral nerve damage, damaged and undamaged neighbouring afferents experience altered exposure to neurotrophic factors, and this could account for some of the changes in afferent properties observed (Millan, 1999, Pezet & McMahon, 2006). Neurotrophic factors are produced by target tissues and act on receptors expressed on the terminals of sensory neurons to cause both peripheral effects and a retrograde signal that travels to the neuronal cell body to exert transcriptional control (Lindsay & Harmar, 1989, Pezet & McMahon, 2006). Axonal denervation such as occurs distal to nerve injury disrupts the access of neurotrophic factors to afferent cell bodies, and thereby disrupts transcriptional regulation (Pezet & McMahon, 2006). Evidence suggests that such disruption may significantly contribute to neuropathy-induced abnormalities. Intrathecal infusion of NGF prevented axotomy-induced changes in the levels of the neuropeptides SP, CGRP,

VIP and galanin (Averill et al., 2004), and decreased behavioural hyperalgesia in the CCI model (Ren et al., 1995). Similarly, intrathecal infusion of the neurotrophic factor GDNF reduced neuropathic pain behaviours following PNL, and reduced the spontaneous activity recorded in L4 and L5 DRG neurons, and alterations in Na^+ channel expression observed following SNL (Boucher et al., 2000).

In parallel with this loss of neurotrophic factor access, the remaining spared axons may have access to increased levels of neurotrophic factors due to decreased competition, and this increased access could affect the properties of the spared neurons. Therefore, the altered exposure to neurotrophic factors of both damaged and spared axons may play a role in the alterations in protein expression and afferent properties observed following peripheral nerve damage.

1.15 Central spinal cord changes in neuropathic pain

Significant changes in pain processing occur in the spinal cord occur following peripheral nerve damage, principally including central sensitisation of dorsal horn neuronal responsiveness, and decreased inhibitory input.

1.15.1 Central sensitisation in neuropathic pain

Synaptic plasticity, the modification of synaptic strength and structure, is a fundamental property of the nervous system. Increased input to the spinal cord results in a facilitation of synaptic transmission, termed central sensitisation (Ji et al., 2003; Wall & Woolf, 1986; Woolf, 1983). The classical definition of central sensitisation is an increase in the excitability of dorsal horn neurons, such that their firing threshold is decreased and receptive field area is increased, following and outlasting, a short barrage of nociceptor input (Cook et al., 1987; Woolf & Wall, 1986; Woolf, 1983). Most input to dorsal horn neurons is subthreshold, and does not evoke an action potential output (Woolf & King, 1990). However, after a brief (~20 second) period of stimulation of afferents at intensities sufficient to activate nociceptors, increased synaptic efficacy allows dorsal horn neurons to be activated by previously subthreshold input (Woolf & King, 1990). Thus the dorsal horn neurons have become sensitised, allowing activation by lower inputs. In neuropathic

pain states, it is thought that central sensitisation may be driven by continuous input from spontaneously firing afferents (Ji et al., 2003).

Central sensitisation is generated by input from nociceptive fibres and includes a potentiation of this input, but can also include a potentiation of synapses made by Aβ fibres onto dorsal horn neurons, a phenomenon which is classified as heterosynaptic facilitation (Garry & Fleetwood-Walker, 2004a,b; Ji et al., 2003; Woolf & King, 1990). This may allow these low-threshold fibres to drive nociceptive dorsal horn neurons, and may thereby account for the phenomenon of allodynia, in which low-threshold touch is perceived as noxious (Ji et al., 2003).

A key example of dorsal horn neuronal sensitisation is the experimental phenomenon of "wind-up", a frequency-dependent facilitation of dorsal horn neuronal responses caused by repeated C fibre input. Wind-up is observed as a gradual increase in the neuronal response to C fibre input, with continued firing up to several minutes following the cessation of stimulation (Dickenson & Sullivan, 1987; Mendell, 1966). Wind-up is observed particularly in deeper WDR neurons rather than superficial neurons and may therefore particularly account for sensitisation of these neurons (Dickenson, 1990; Seagrove et al., 2004).

Activity-dependent sensitisation has been reported in rodent, cat and primate dorsal horn neurons (Dougherty & Willis, 1992; Kenshalo, 1982). A central sensitisation-like phenomenon can also be generated in the RVM, anterior cingulate cortex, and amygdala (Neugebauer et al, 2003; Porreca et al 2002; Wei & Zhuo 2001), demonstrating that pain areas beyond the spinal cord show the capacity for sensitisation to their inputs.

Underlying the synaptic facilitation of central sensitisation are changes in ion channel and receptor activity including both short-term post-translational changes, such as the phosphorylation of glutamate receptors and altered trafficking of receptors to the membrane, and long-term transcriptional changes (Ji et al., 2003; Kawasaki et al., 2004; Li et al., 1999; Woolf & Salter, 2000). As is discussed below, glutamate receptors, and signalling pathways engaged downstream of glutamate receptor activation, play a key role in the generation of central sensitisation.

1.15.2 NMDARs in neuropathic pain

The NMDAR only opens in the presence of both glutamate and depolarisation-induced removal of its Mg^{2+} block. Increased input from afferents following nerve or tissue injury acts to remove the Mg^{2+} block, allowing glutamate released from afferents to activate NMDARs, resulting in Ca^{2+} entry and consequently in medium and long-term changes. NMDARs are crucial to the sensitisation of spinal neurons that occurs following injury (Ji & Woolf, 2001). NMDAR antagonists reduce wind-up, but not the initial response itself, of dorsal horn neurons following repeated C fibre stimulation or mustard oil application, demonstrating a selective involvement in neuronal sensitisation (Davies & Lodge, 1987, Dickenson & Sullivan, 1987, Dickenson & Aydar, 1991, Woolf & Thompson, 1991). Behavioural experiments also show an important role for the NMDAR in hypersensitivity. Intrathecally administered NMDA causes behavioural sensitisation in normal animals (Aanonsen & Wilcox, 1987; results section 4.10), and NMDAR antagonists attenuate neuropathic pain behaviours (Chaplan et al., 1997; Mao et al., 1992, 1993; Tal & Bennett, 1993).

NMDAR activation leads to Ca^{2+} entry and subsequent activation of downstream signalling pathways such as the kinases PKC (Vaccarino et al., 1987), Src (Platenik et al., 2000; Salter et al., 1998), and Ca^{2+}/calmodulin dependent protein kinase II (CaMKII) (Bayer et al., 2001; Garry et al., 2003a). NMDAR activation is also linked to activation of the extracellular signal related kinase – mitogen activated protein kinase (ERK-MAPK) pathway, as in hippocampal neurons glutamate induces ERK activation, and this effect is blocked by NMDAR antagonists (Bading & Greenberg, 1991). Activation of these kinases results in changes in the phosphorylation state of certain proteins, thereby affecting their function; and can also lead to activation of transcription factors (Ji et al., 2003). Examples of such transcription factors are members of the CREB (cAMP response element binding protein) family, which can be activated by multiple signalling pathways, including the PKA, PKC, CaMKII and ERK-MAPK signalling pathways (Lonze & Ginty, 2002). Increases in phosphorylation of CREB in the spinal cord are observed following partial sciatic nerve ligation (Ma & Quirion, 2001) and following CCI (Miletic et al, 2002), which can be reversed with inhibitors of PKA, PKC or ERK (Miyabe & Miletic, 2005).

Transcriptional changes induced through factors such as CREB are likely to underlie some of the long-term changes involved in chronic pain states.

1.15.3 AMPARs in neuropathic pain

In addition to a role in mediating acute nociceptive responses, AMPARs are implicated in sensitisation. Intrathecal application of AMPAR blockers attenuated behavioural sensitisation following CCI, at doses that had no effect on contralateral or naïve reflex sensitivity (Garry et al., 2003b). This perhaps indicates an increased sensitivity of AMPARs in neuropathic states, so that low doses of AMPAR antagonists can attenuate sensitisation without affecting normal responses.

As AMPARs mediate the majority of glutamate-induced excitatory current in the dorsal horn as elsewhere in the nervous system (Dougherty et al., 1992; Ehlers, 2000; Kandel & Siegelbaum, 2000; Malenka & Nicoll, 1999), modulation of AMPAR expression will have profound consequences for glutamatergic transmission. Increased overall expression of the AMPAR GluR1 subunit (Harris et al., 1996) and of the GluR2 subunit (Garry et al., 2003b; Harris et al., 1996) has been observed in models of neuropathic pain, suggesting an increased AMPAR current in such states, which may contribute to increased spinal neuron excitability.

1.15.4 Decreased inhibition in neuropathic pain

In addition to the facilitation of excitatory glutamatergic transmission described above, there is evidence for a decrease in endogenous inhibitory mechanisms in neuropathic pain states, which may contribute overall increased excitability.

Evidence for a decrease in GABAergic transmission

Spinal cord levels of GABA and of GABA-B receptors are reduced following peripheral axotomy (Castro-Lopes et al., 1993, 1995), and levels of the GABA synthesising enzyme glutamic acid decarboxylase-65 kDa are decreased ipsilateral to CCI or SNI injury (Moore et al., 2002), suggesting a decrease in GABAergic transmission in neuropathic states. In CCI and SNI models, the incidence and magnitude of afferent-evoked IPSCs were substantially reduced, particularly the bicuculline-sensitive component (Moore et al., 2002), suggesting that the decrease in GABA levels is reflected in a functional decrease in GABAergic transmission.

However, another study showed no change or indeed a slight increase in the GABAergic inhibitory tone, indicated by an increased effect of bicuculline, in the SNL model (Kontinen et al., 2001), and other studies have seen no change in GABA-immunoreactivity following CCI (Polgar et al., 2003). Therefore it is not clear to what extent a decrease in GABAergic transmission contributes to spinal cord hyperexcitability in neuropathic pain states.

Evidence for a decrease in spinal opioid transmission

In animals with a peripheral nerve injury, higher doses of intrathecal morphine are required to produce behavioural analgesia (Bian et al., 1995; Dickenson & Suzuki, 2005), and there is a reduced inhibitory effect of systemic morphine on the responses of spinal neurons (Dickenson & Suzuki, 2005; Matthews & Dickenson, 2002; Ossipov et al., 1995), in comparison with normal animals, suggesting a decrease in spinal cord opioidergic transmission in neuropathic states. For many years the clinical consensus was that opioids were ineffective in neuropathic pain (Arner & Meyerson, 1988), but higher doses of opioids have been shown to be clinically effective and are recommended for neuropathic pain (Moulin, 2006), in line with the animal studies which demonstrate an inhibitory effect of opioids, albeit reduced, in nerve-injury models.

Opioid receptor binding sites and μ-opioid receptor-immunoreactivity are decreased in animal models of neuropathic pain (Besse et al., 1992; de Groot et al., 1997; Porreca et al., 1998). Therefore the decreased sensitivity to intrathecal opioids observed behaviourally (Bian et al., 1995) may be due to decreased spinal expression of opioid receptors. Another reason underlying this decreased efficacy may be an increase in levels of the neuropeptide CCK and its receptors. Intrathecally administered CCK reduces the analgesic effect of exogenous opioids, an effect that is blocked by antagonists or antisense knockdown of the CCK-2 receptor (Cesselin, 1995; Vanderah et al., 1994; Watkins et al., 1985; Wiesenfeld-Hallin et al., 1999, 2002), indicating an antagonistic effect of CCK on opioidergic transmission. Following peripheral nerve injury, mRNA levels for both CCK and the CCK-2 receptor are increased in primary afferent neurons (Hokfelt et al., 1994; Wiesenfeld-Hallin et al., 2002; Xu et al., 1993), which may result in increased inhibition of opioidergic transmission. In support of this, intrathecal administration of CCK-2

antagonists restored opioid efficacy in the SNL model (Nichols et al., 1996) and axotomy (Xu et al., 1993) models of neuropathy.

1.16 Historical background of cooling-induced analgesia

The first record of cold as an analgesic therapy was left by the father of medicine, Hippocrates (Figure 1.4), who recommended application of cold water for the treatment of bruises, joint pains and ulcerations (Adams, 2006; Sprengell, 1735). Similar uses are recorded by Galen and other eminent early physicians (Siegel, 1970). This traditional therapy is still found useful today, with clinical trials showing beneficial effects of cooling on chronic back pain, dental pain, post-operative pain, and muscle injuries (Cohn et al., 1989; Levy & Marmar, 1993; Melzack et al., 1980a,b; Ross & Soltes, 1995; Scheffler et al, 1992; review by Sauls, 1999). Nursing practice reports encourage the use of cold as an adjunctive therapy to be used alongside pharmacological interventions (McCaffery, 1980; 1990) and cold sprays and gels for muscle and joint pain have been used to some effect in general practice and sport (Traherne, 1962). Therefore local cooling has been shown to be an effective analgesic in a variety of pain states, including both chronic states such as chronic back pain (Melzack et al., 1980a) and acute states, as in the use of cold sprays in sport (Traherne, 1962). Animal experiments show an analgesic effect of cool environmental temperatures on nociceptive thresholds (Osgood et al., 1990; Schoenfeld et al., 1985) and a similar effect has been demonstrated psychophysically in humans (Hardy et al., 1951; Strigo et al., 2000). Studies on pain thresholds in humans using microelectrode stimulation to evoke pain demonstrate an analgesic effect of local cooling (Bini et al, 1983; Bugaj, 1975; Miller & Weber, 1990; Saeki, 2002). Cutaneous cooling inhibits nociceptor-driven dorsal horn neuron firing (Kanui, 1985), demonstrating an effect on spinal cord nociceptive processing. Therefore there is significant clinical and experimental evidence for an analgesic effect of local cutaneous cooling. It should be noted that a variety of cooling stimuli have been used in the studies described, from topically applied ice to ether evaporation, and the resulting skin temperature rarely measured, and therefore it is difficult to state precisely the temperatures that evoked analgesia in these studies.

Cooling-induced analgesia appears to be at least partially mediated centrally, as local cutaneous cooling of the skin (from 30 to 20°C) prevents pain produced by downstream intraneural stimulation of afferent neurons in humans (Bini et al, 1984; Bugaj, 1975).

1.17 Menthol and analgesia

Menthol and menthol-containing mint oils produce a sensation of cooling (Green, 1986, Green, 1992), and menthol is known to activate cold-sensitive afferents and cold-sensitive receptors (Hensel & Zotterman, 1951, Peier et al., 2002b, McKemy et al., 2002). Preparations containing menthol or mint oils have, like physical cooling, been shown to have analgesic effects and menthol is a common ingredient in over-the-counter pain relief remedies. In humans, topical mint oil alleviated experimentally thermally-elicited pain (Gobel et al., 1994) and application of menthol caused a short-term analgesia in the mouth against pain elicited by oral capsaicin (Green & McAuliffe, 2000). In animals, systemic administration of (-)-menthol increased pain thresholds in the mouse hot-plate and acetic acid writhing tests (Galeotti et al., 2002), and co-injection of menthol attenuated the licking and shaking behaviour elicited by intraplantar injection of capsaicin (Premkumar et al., 2005). Notably, menthol and mint oils appear effective against pain of neuropathic origin. Topical mint oil is used to relieve neuralgia in both traditional Chinese and European herbal medicine (Wright, 1870, Blumenthal et al., 1998). Recently, effective clinical treatment of a case of postherpetic neuralgia by application of topical peppermint oil (containing 10% menthol) was reported (Davies et al., 2002). The cooling effect of menthol, together with the fact that both menthol and cool temperatures can produce analgesia, suggests the possibility that cooling and menthol produce analgesia through a common mechanism.

Ancient Analgesia

Figure 1.4 Historical Analgesia
The father of medicine, Hippocrates, recommended cold for pain relief in his writings, and mint is used in traditional Chinese and European medicine for relief of neuralgia. Images courtesy of the Wellcome Trust History of Medicine Library.

1.18 Sensory detection of cold

In order to understand how topical cooling may produce a centrally-mediated analgesia, it is necessary to consider how cold is detected and processed by the somatosensory system. In vivo recording studies of afferents, first carried out in the 1950s, identified populations of cold-sensitive sensory neurons, and further electrophysiological studies have examined spinal cord neurons responding to cold. Studies on cultured DRG and trigeminal ganglion cells have continued this study of cold-sensitive neurons, and pointed towards possible transduction mechanisms underlying cold-sensitivity. Recently, molecular biology has enabled the identification of specific proteins which act as cold-sensitive ion channels.

1.19 In vivo recordings of cold-sensitive afferents

Cold-responsive primary afferents have been recorded in man, primates, rodents, cats and dogs. They fall into two loose groupings – fibres activated by low threshold "cool" temperatures (responding to skin temperatures between ~15-30°C) (Darian-Smith et al, 1973; Dubner et al, 1975; Kenshalo & Duclaux, 1977), and fibres responding to high-threshold (intense) cold temperatures (under 15°C) (Georgopoulos, 1977; LaMotte & Thalhammer, 1982).

It is difficult to collate the data to form a precise categorisation of cold-sensitive afferents. Recordings have been performed in different species and in different nerves, and these different preparations may show significant differences in the neuronal populations that respond to cold – for example, Aδ fibres comprise a much higher proportion of cold receptors in primates than in rodents (Iggo, 1969). Different studies have used different protocols to stimulate cold-sensitive fibres, such as a Peltier device, or application of ice. In general, cold-sensitive neurons constitute a low proportion of the total number of neurons in a nerve, and therefore most studies generate much lower numbers than comparable studies on for example mechanoreceptors, further complicating data interpretation.

1.19.1 Low-threshold cold-sensitive afferents

Low-threshold cold-sensitive - "LTcold" - afferents show steady state discharges to skin temperatures between 15 and ~30°C, temperatures which evoke a sensation of innocuous cooling (Croze & Duclaux, 1978; Chen et al., 1996; Stevens, 1979). LTcold afferents exhibit spontaneous activity at normal skin temperature (Hensel & Zotterman, 1951). LTcold afferents generally respond specifically to cold and cooling and are insensitive to other stimuli (Kenshalo & Duclaux, 1977; Fang et al., 2005; Hensel et al., 1960; Hensel & Iggo, 1971; Iggo, 1969). Recordings of afferents show that afferents respond maximally at particular static temperatures, with a gradual decrease in firing frequency either side of this preferred temperature (Iggo, 1969). These afferents discharge maximally to temperatures between ~20 and 35°C (Darian-Smith et al., 1973; Dubner et al., 1975; Iggo, 1969; Iriuchijima & Zotterman, 1960; Kenshalo & Duclaux, 1977). Afferents show a greater dynamic than static range, in that they may be excited by decreases in temperature outside of their static range (by approximately 5°C) (Darian-Smith et al., 1973; Iggo, 1969; Kenshalo & Duclaux, 1977), and also respond more vigorously (with a higher firing frequency) to a decrease to a particular temperature, than they do to the static temperature (Dubner et al., 1975; Kenshalo & Duclaux, 1977; Iggo, 1969). A proportion of cold-sensitive neurons have also been identified to respond to strong heating (Campero et al., 2001; Dodt & Zottermann, 1952; Long, 1977), which may underlie the psychophysical phenomenon of paradoxical cold sensation, in which strong heat applied to a spot on the skin innervated specifically by a cold-sensitive neuron elicits a sensation of cold, rather than heat (Norrsell et al., 1999).

In primate glabrous skin, LTcold afferents appear to be exclusively Aδ fibre neurons (Darian-Smith et al., 1973; Hensel & Iggo, 1971; Iggo, 1969), but both Aδ and C fibre LTcold afferents have been found in hairy skin (Iggo, 1969). LTcold afferents with C fibre conduction velocities have also been recorded in microneurographic studies in humans (Campero et al., 2001). LTcold afferents appear to be exclusively C fibre neurons in rodents (Fang et al., 2005; Iggo, 1969; Iriuchijima & Zotterman, 1960; Lynn & Carpenter, 1982), and in cats (Bessou & Perl, 1969; Hensel et al., 1960; Jiang et al., 2002). It is generally agreed that LTcold-sensitive afferents comprise a relatively low proportion of the total nerve. Studies have shown between 3 and 6% of C fibres responsive to small decreases in

temperature in a range from 32 – 20°C in recordings of rat saphenous nerve (Lynn & Carpenter, 1982), rat lumbar DRG (Fang et al., 2005), cat femoral nerve (Bessou & Perl, 1969), and a variety of nerves in monkey hairy skin (Treede et al., 1992). However, an extensive study of the rat sural and plantar nerves identified a significantly higher proportion of LTcold C fibre neurons, constituting 21% and 23% of rat sural and plantar nerves respectively. This study also found 5% of Aδ afferents in the sural nerve were sensitive to cool temperatures (Leem et al., 1993), although slightly lower stimulation temperatures were used (in a range of 32 to 17°C). In vivo recordings of cool-responsive DRG C fibre neurons showed that these had higher conduction velocities and shorter action potential durations compared with polymodal C nociceptors (Fang et al., 2005) indicating that even though LTcold afferents are C fibre neurons, they show properties distinct from those of nociceptive neurons.

1.19.2 High-threshold cold-sensitive afferents

Cooling the skin to temperatures below 15°C produces sensations of pain in humans (Chery-Croze & Duclaux, 1980; Chery-Croze, 1983; Wolf & Hardy, 1941; Yarnitsky & Ochoa 1990). Afferents responding to temperatures below 15°C, described here as high-threshold cold-sensitive (HTcold) afferents, have also been described in in vivo fibre recordings in primates (Georgopoulos, 1977; Iggo & Ogawa, 1971; LaMotte & Thalhammer, 1982), cats (Iggo, 1959) and rodents (Bessou & Perl, 1969; Koltzenburg et al., 1997; Leem et al., 1993; Lynn & Carpenter, 1982; Simone & Kajander, 1997). Similarly to LTcold afferents, HTcold afferents fire at particular static temperatures, but show an increased discharge rate to a decrease in temperature (Iggo, 1959). Many studies also describe HTcold afferents as responsive to high-threshold mechanical stimuli, suggesting that they are polymodal nociceptive afferents, although frequently only the response to mechanical stimuli has been measured (Georgopoulos, 1977; Iggo & Ogawa, 1971; Koltzenburg et al., 1997; Lynn & Carpenter, 1982; Leem et al., 1993). In primates, HTcold responses are associated with both Aδ and C fibres (Georgopoulos, 1977; Iggo & Ogawa, 1971; LaMotte & Thalhammer, 1982). In cats and rodents, initial studies identified HTcold afferents with C fibres (Burgess & Perl, 1967; Iggo, 1959; Lynn & Carpenter, 1982).

However, further studies have also identified Aδ fibre HTcold afferents in rodents (Koltzenburg et al., 1997; Leem et al., 1993; Simone & Kajander, 1997). Varying proportions of neurons responding to HTcold temperatures have been found in rodents. One study found that 11% of Aδ nociceptors responded to lowering the skin temperature to 10°C, but that by 0°C this had increased to 30%, and by -18°C to 100% of Aδ nociceptors (Simone & Kajander, 1997), although it is possible that the nociceptive responses in this study were due to damage associated with freezing at these very low temperatures. Other studies have shown that approximately 10% of Aδ nociceptors are sensitive to stimuli below 12°C (Fang et al., 2005; Koltzenburg et al., 1997; Leem et al., 1993). Estimates of C polymodal nociceptors responsive to temperatures below 12°C have varied between 3.5% to 30% (Bessou & Perl, 1969; Fang et al., 2005; Koltzenburg et al., 1997; Leem et al., 1993). Discrepancies may be due to differences in protocols or differences between recording sites: for example Leem et al. found 8% of C fibres in the plantar nerve were cold sensitive, but only 3.5% in sural nerve.

1.20 Cold-sensitive dorsal horn neurons

Noxious and innocuous cold signals also appear to be processed differently in the superficial dorsal horn. In both primates and cats, neurons responding exclusively to innocuous cool temperatures applied to the skin (temperatures between 34 and 15°C) have been recorded in LI of the dorsal horn (Burton, 1975; Craig et al., 2001; Han et al., 1998; Iggo & Ramsey, 1974, Mokha, 1993). The population of nociceptive specific neurons in LI, which are activated by noxious heat and pinch stimuli (Christensen and Perl, 1970) include a subpopulation of neurons that respond to noxious cold (Craig et al., 2001; Han et al., 1998). Cold-sensitive nociceptive-specific cells responded to temperatures between 24°C to 9°C, so there is some overlap in the temperature ranges of these two types of neurons (Craig et al., 2001). Although a detailed analysis of the distribution of these cells has not been attempted, cold-specific cells comprised 28% and cold-sensitive nociceptive specific cells comprised 15% of labelled LI cells in the cat (Han et al., 1998). In the cat, the morphology of cold-specific cells was distinctive from that of the nociceptive-

specific cells, the former showing a pyramidal structure and the latter a multipolar structure (Han et al., 1998). Cold-specific cells and the nociceptive-specific cells also showed different conduction velocities, different spontaneous discharge rates and different thalamic terminations (Craig et al., 2001; Craig & Dostrovsky, 2001), suggesting that these are two fundamentally different types of cells. Therefore innocuous cool and noxious cold input appears to be processed differently in the dorsal horn.

1.21 Cold transduction in sensory neuron cultures

The fine structure of peripheral sensory neurons presents too great technical difficulties for electrophysiological study of peripheral transduction processes. In contrast, the cell soma can easily be recorded from with microelectrodes. When dissected from its axon and cellular neighbours and cultured, the cell soma appears to display many of the functional processes assumed or known to occur at the peripheral terminal (Cesare et al., 1996; Gold et al., 1996; LaMotte, 2006). Therefore, recordings from the somata of isolated DRG and TG neurons have been used as a model of peripheral sensory neurons to study transduction processes.

Experiments in sensory neuron cultures seem to indicate two broad populations of cold-sensitive neurons; one which shows activation thresholds around 30°C, and one with lower activation thresholds of around 20°C (Babes et al., 2004; Nealen et al., 2003; Thut et al., 2003). These populations resemble the LTcold and HTcold afferents observed in in vivo afferent recordings, supporting a correlation between the properties of sensory cultures and neurons in vivo. A key property frequently found in cold-sensitive neurons is sensitivity to menthol (Reid et al., 2002; Thut et al., 2003). Classic work by Hensel and Zotterman (Hensel & Zotterman, 1951) demonstrated that menthol potentiates the response of trigeminal fibres to cold, and proposed the existence of specific cold receptors, which are also menthol-sensitive. The identification in cultured DRG neurons of an inward Ca^{2+}-permeable cationic current that is activated by cooling (with an average threshold of 30°C), and is also activated and potentiated by menthol, suggests a common transduction mechanism that underlies the neuronal response to low threshold cold temperatures and menthol

(Reid & Flonta, 2001a; Reid et al., 2002). Such a mechanism has since been discovered: the TRPM8 (TRP-melastatin-family-member 8) receptor, which shows remarkably similar properties to the native cold-and-menthol-sensitive current.

1.22 Cold-sensitive ion channels and their potential role in cooling-induced analgesia

In recent years ion channels such as TRPM8 have been discovered that are activated by temperatures in the range for the physiological detection of cold, and that therefore represent candidates for the physiological molecular receptors of cold temperatures and cooling. The identification of such ion channels allows for the first time study of molecular mechanisms underlying cooling-induced analgesia. The most extensively studied cold receptors to date are TRPM8 and TRPA1 (TRP-ankyrin family member 1), which are both members of the TRP channel family. Other identified cold-sensitive channels include the 2-pore domain K^+ channels TREK-1, TREK-2 and TRAAK, and the epithelial sodium channel ENaC (Askwith et al., 2001; Kang et al., 2005; Maingret et al., 2001); these are discussed further in section 1.26. Cold-activated conductances have been described in sensory neurons which do not match the properties of TRPM8 or TRPA1, supporting the existence of alternative cold transduction mechanisms (Babes et al., 2004; Madrid et al., 2006; Munns et al., 2006). At present, however, the alternative channels and cold-activated conductances described are too poorly defined to allow investigation of their precise role in cooling-induced analgesia, and therefore I have concentrated on the cold-sensitive TRP channels. I have focussed on TRPM8 as the most promising candidate for a molecular mediator of cooling-induced analgesia. This is because TRPM8 responds to moderate cooling and to menthol, which has also been shown to have an analgesic action, and therefore TRPM8 may underlie the analgesic actions of both cooling and menthol. TRPA1 appeared a less promising candidate, firstly because there is uncertainty as to whether it does indeed respond to cold (Bautista et al., 2006; Jordt et al., 2004; Nagata et al., 2005), and secondly because it is activated by noxious chemicals, application of which have been shown to produce hyperalgesia in animals (Bandell et al., 2004) and pain sensations in man (Namer et al., 2005), and is

expressed in apparently polymodal nociceptive populations, suggesting that it may be primarily involved in nociception (as is discussed further below). Therefore these studies have concentrated on TRPM8, rather than TRPA1.

The following sections will discuss: the TRP family in general; TRPM8 in detail, as the main focus of these studies; TRPA1, and its potential role in cold sensation; and finally current knowledge of non-TRP mediators of cold transduction.

1.23 The TRP family of cation channels

TRP channels constitute an ancient and diverse family of non-selective cation channels. The first TRP channels were discovered for their role in Drosophila phototransduction (Montell & Rubin, 1989); TRPs have since been identified in eukaryotes from yeast to mammals (Ramsey et al., 2006), and show a remarkably diverse range of properties and functions, from Mg^{2+} homoeostasis to nociception. Mammalian TRPs are divided into subfamilies based on sequence homology: the TRPC (canonical); TRPV (vanilloid - so named because the first identified member is activated by the vanilloid capsaicin); TRPM (melastatin, named for the role of the first member of this family in melanoma metastasis); TRPA (ankyrin, named for the 14 predicted ankyrin domains in the N-terminal tail of this subfamily member); TRPP (polycystin, named for involvement in polycystic kidney disease); and TRPML (mucolipin, mutations in this channel are associated with mucolipidosis) subfamilies (Clapham, 2003; Moran et al., 2004; Ramsey et al., 2006). Figure 1.5 shows this classification based on the homology of their DNA sequences. The genetic sequences and structures of TRP channels are so diverse that the only similarity shared by all identified TRPs is a structure based on six transmembrane (TM) domains (Clapham et al., 2003; Ramsey et al., 2006). The TRP channels therefore belong to the larger superfamily of six transmembrane domain cation channels.

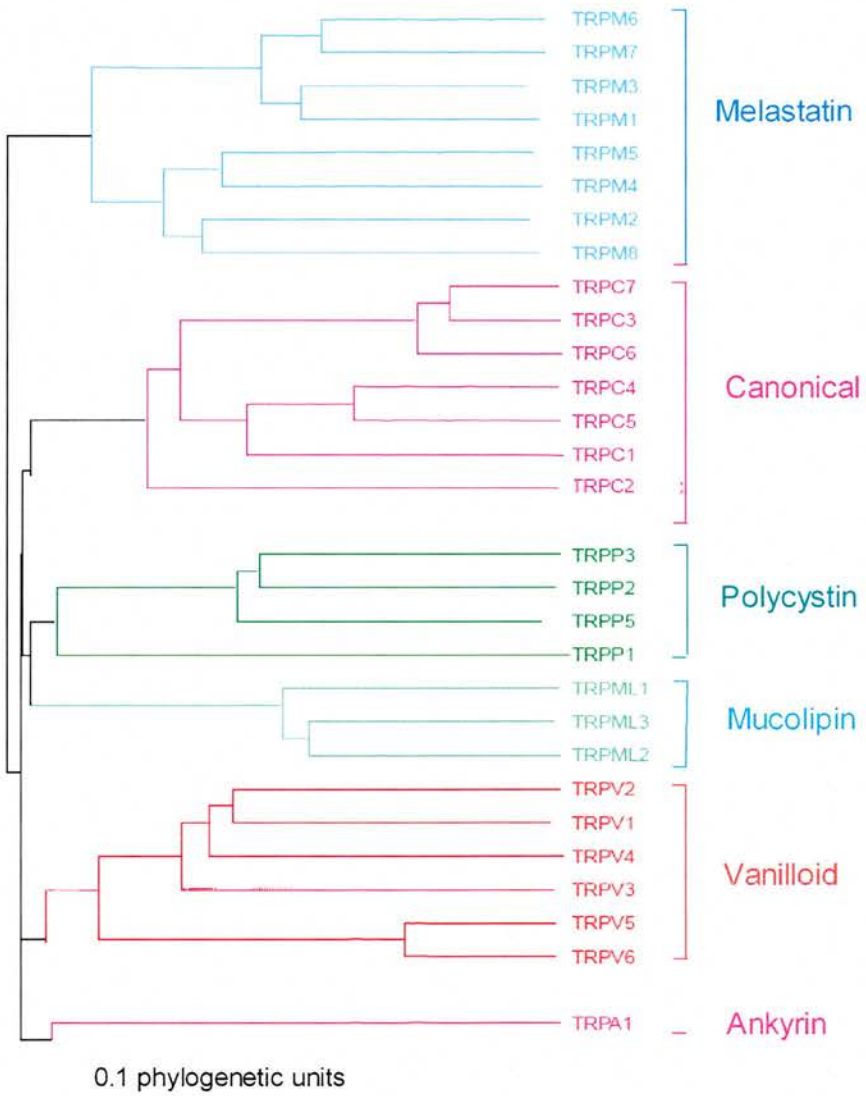


Figure 1.5 The TRP channel family
Figure shows the varied members of the TRP channel family based on sequence homology. Figure adapted from Moran et al., 2004.

1.24 TRPM8

The TRPM8 protein was originally identified as a Ca^{2+} channel in prostate tissue and named Trp-p8 (Tsavaler et al., 2001). The cloning and identification of TRPM8 as a cold-sensitive ion channel was accomplished in 2002 by two separate groups (McKemy et al., 2002; Peier et al., 2002b). As described above, work on trigeminal fibres and cultured DRG neurons identified a current that could be activated by cold and menthol (Hensel & Zotterman, 1951; Reid & Flonta, 2001a). McKemy et al. used this information to isolate the TRPM8 channel by screening a cDNA library for menthol-induced elevation of cytosolic Ca^{2+}, measured by fluorescence, a method analogous to that used to clone TRPV1 (Caterina et al., 1997; McKemy et al., 2002). Peier et al. used a strikingly different method, searching human genomic databases with a hidden Markov model constructed from TRP proteins of different species to identify exon sequences similar to TRPV1. Based on this information, primers were designed and used on newborn mouse DRG tissue to amplify a fragment of a mouse homologue of a TRP channel by RT-PCR (reverse transcriptase-polymerase chain reaction). From this initial sequence and exon prediction programs, rapid-amplification-of-cDNA-ends-PCR (RACE-PCR) was used to obtain the 5' and 3' ends of TRPM8. Thus the entire TRPM8 gene was cloned and sequenced, and was then expressed in cells and characterised as a cold-sensitive receptor (Peier et al., 2002b). TRPM8 is part of the TRPM subfamily of the TRP family, based on amino acid sequence homology.

1.24.1 Temperature sensitivity of TRPM8

TRPM8 is activated by cool temperatures and cooling chemicals such as menthol and icilin (McKemy et al., 2002; Peier et al., 2002b) and carries a non-selective cation current, similarly to other TRP channels. When TRPM8 is expressed in heterologous systems, the mean activation threshold is between 19-25°C, with increasing activation as temperature is decreased further, saturating at around 10°C, demonstrating that TRPM8 is active within the range of both innocuous and noxious cold temperatures (de la Pena et al., 2005; McKemy et al., 2002; Peier et al., 2002b). The temperature activation threshold of native sensory neurons that are activated by

menthol and cooling and that are inhibited by the non-selective TRP channel antagonist BCTC (N-(4-tertiarybutylphenyl)-4-(3-chloropyridin-2-yl)-tetrahydropyrazine-1(2H)-carboxamide) is somewhat higher: around 28-30°C (Madrid et al., 2006; Nealen et al., 2003; Reid, 2005), suggesting that TRPM8 alone may not fully account for the native cold-and-menthol-sensitive current. It is possible that TRPM8 sensitivity may be modulated in sensory neurons by intracellular signalling molecules, or by interaction with sensory neuron-specific scaffolding proteins, as occurs for TRP channels involved in Drosophila phototransduction (de la Pena et al., 2005; Montell, 1998). In support of such a modulation of TRPM8 function by intrinsic cellular proteins or pathways, recording from excised patches in native sensory cells shifts the temperature threshold of the native cold-and-menthol current to lower temperatures, by as much as 13°C (Reid & Flonta, 2002). Alternatively, the native TRPM8 could exist as a heteromultimer of TRPM8 assembled with subunits of other ion channels, or an assembly of different splice variants of TRPM8, which could alter the temperature sensitivity of the resultant channel (de la Pena et al., 2005; Reid, 2005). However, the temperature-sensitive TRPV channels preferentially assemble as homomers (Hellwig et al., 2005) and certainly heteromeric assembly of TRPM8 has not been demonstrated (Schaefer, 2005). Alternatively, the cold threshold of native TRPM8-expressing sensory neurons may be the result of combined expression of TRPM8 with additional cold-sensitive ion channels (de la Pena et al., 2005; Reid, 2005).

The mean threshold of 30°C of the TRPM8-like cold-and-menthol-sensitive current in sensory neurons is consistent with the thresholds of LTcold-sensitive neurons recorded in vivo (Iggo, 1969; Reid & Flonta, 2001a; Reid, 2005), suggesting a role for TRPM8 in innocuous cold transduction. However, TRPM8 is still active at temperatures below the 15°C psychophysical threshold for noxious cold sensation, saturating at around 10°C (McKemy et al., 2002; Peier et al., 2002b), and so could also play a role in noxious cold sensitivity.

1.24.2 Chemical sensitivity of TRPM8

TRPM8 is activated by chemicals that elicit the subjective experience of coolness including menthol, icilin (Wei & Seid, 1983), eucalyptol, and a range of Wilkinson

Sword manufactured chemicals (Behrendt et al., 2004). Chemical agonists such as these also result in a functional sensitisation of TRPM8, shifting the activation threshold to higher temperatures (McKemy et al., 2002; Peier et al., 2002b), demonstrating co-operativity between temperature and chemical-based stimuli. The effect on TRPM8 of various agonists is shown in Figure 1.6. Of these chemicals, icilin and menthol are the most comprehensively studied agonists. The effect of menthol on cool fibres is stereoselective, with (-)-menthol exerting a stronger effect than (+)-menthol (Schafer et al., 1986). Similarly, (-)-menthol has a higher potency acting at TRPM8 than (+)-menthol (Behrendt et al., 2004). Icilin (Wei & Seid, 1983), which is structurally unrelated to menthol, was until recently the most effective TRPM8 agonist known, acting at TRPM8 with about 2.5-fold greater efficacy and nearly 200-fold greater potency than menthol (McKemy et al., 2002). Very recently, a report found that the Wilkinson Sword-developed cooling chemical WS-12 had an even higher affinity for TRPM8 than icilin, with an EC_{50} of 39 nM compared with 500 nM for icilin in similar conditions (Beck et al., 2006), and may therefore be a promising agent for future studies of TRPM8.

Interestingly, systemic (intraperitoneal) administration of icilin (doses of ~ 0.5mg/kg or ~ 500 nmol per average rat) in rats evokes vigorous shaking known as "wet-dog shakes" (Wei & Seid, 1983), a phenomenon similar to that seen following morphine withdrawal (Collier et al., 1974). This shaking has been reported to be antagonised both by agonists of μ- or κ-opioid receptors acting centrally, but not peripherally (Werkheiser et al., 2006a), and by intracerebroventricular administration of adenosine (Tse & Wei, 1986). Although the mechanism underlying these actions is unclear, these results indicate that the shaking behaviour elicited by icilin is produced centrally in the brain, and therefore appears to be separate from the peripheral action of icilin at the TRPM8 receptor.

Menthol and icilin appear to activate TRPM8 through different mechanisms. In contrast to menthol and cold activation, icilin activates TRPM8 with a variable latency, and has no effect on TRPM8-expressing cells in the absence of extracellular Ca^{2+} (Andersson et al., 2004; Chuang et al., 2004; McKemy et al., 2002). In fact, an increase in intracellular Ca^{2+} is required for icilin activation of TRPM8 (as icilin's action is abolished by chelation of intracellular Ca^{2+}), and it is suggested that icilin

alone can activate TRPM8 to a low level, which allows some Ca^{2+} into the cell, leading to a positive feedback loop and full icilin activation of the channel (Chuang et al., 2004). A further difference is that icilin causes rapid and strong desensitisation of TRPM8 currents, whereas desensitisation to menthol is more variable (Andersson et al., 2004; Chuang et al., 2004; McKemy et al., 2002; Weil et al., 2005). Therefore there are substantial differences between the activation mechanisms of these ligands, and in line with this, sensitivity to these ligands is conferred by different parts of the TRPM8 molecule (see section 1.24.5).

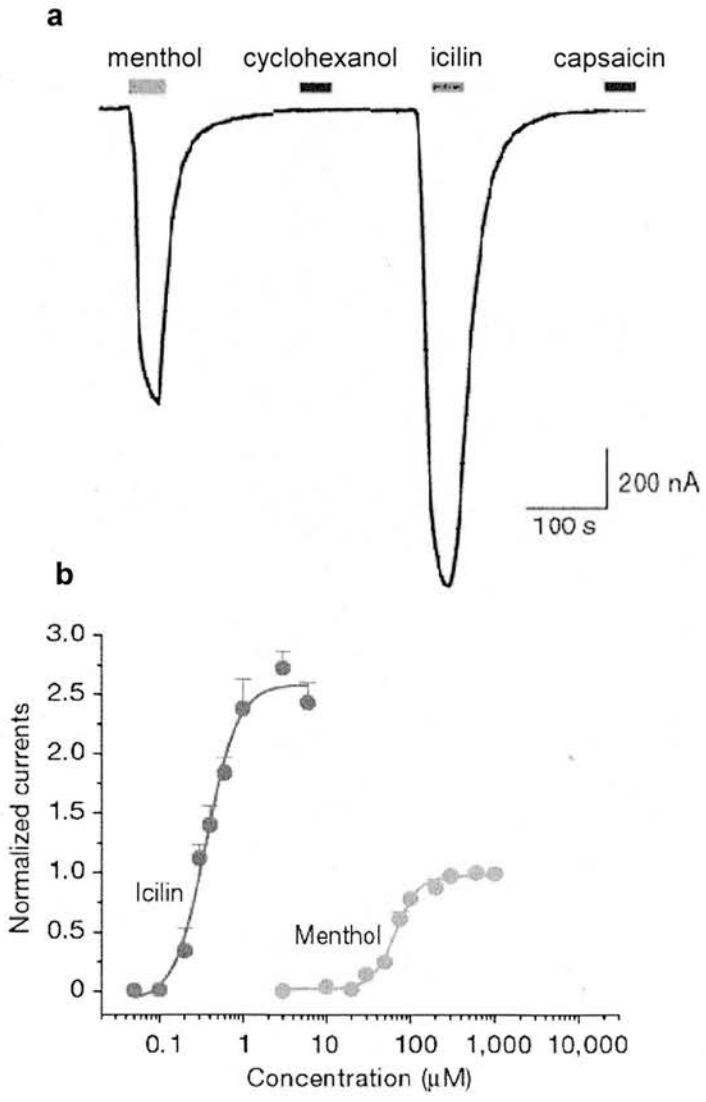


Figure 1.6 Icilin and menthol-evoked currents in TRPM8-expressing cells

a) Currents elicited by menthol (100 μM) cyclohexanol (500 μM, control), icilin (300 nM) and the TRPV1 agonist capsaicin (1 μM) in Xenopus oocytes expressing TRPM8, measured under voltage clamp at -60 mV. Bars denote the duration of agonist application.

b) Concentration-response curves for icilin and menthol, normalised to those responses evoked by 500 μM menthol, shown as mean ± SEM.

Both figures taken from McKemy et al., 2002.

1.24.3 Properties of the TRPM8 current

Other than the difference in threshold discussed above, the properties of cold-and-menthol-sensitive currents in TRPM8-transfected cells match well with currents in native cold-and-menthol-sensitive sensory neurons, and therefore TRPM8 is generally accepted as being the molecular mediator of this current (McKemy, 2005; Reid, 2005). Both the native cold-and-menthol-sensitive current, and currents in TRPM8-transfected cells show a relatively high permeability to Ca^{2+}, and little selectivity among monovalent cations (McKemy et al., 2002; Peier et al., 2002b; Reid et al., 2002; Reid, 2005). In sensory neurons, the majority of the increase in intracellular Ca^{2+} produced by cold or menthol stimulation appears to be a result of depolarisation-induced opening of voltage-gated Ca^{2+} channels, as it is reduced by blocking voltage-gated Ca^{2+} channels with Cd^{2+} (Reid, 2005; Thut et al., 2003), and the majority of depolarisation depends on Na^{+} as it is prevented by replacing Na^{+} in the external solution (Reid et al., 2002; Reid, 2005).

Additional properties demonstrated by the cold/menthol current in native sensory neurons and TRPM8-expressing cells are a steep outward rectification (McKemy et al., 2002; Reid et al., 2002), and a Ca^{2+}-dependent adaptation (McKemy et al., 2002; Peier et al., 2002b; Reid et al., 2002; Reid, 2005; Reid & Flonta, 2001a;). Adaptation is observed as a decline in cold-activated current at steady temperature, with a time constant of approximately 1 minute for both decline and recovery, and a shift in temperature sensitivity, so that the same maximal current may be elicited but requires stronger cooling (Reid et al., 2002; Reid, 2005). Adaptation is absent in excised outside-out patches (Reid & Flonta, 2002), and therefore is not a feature of the channel itself but must involve intracellular processes, and is dependent on Ca^{2+}, as it is prevented by replacing extracellular Ca^{2+} with Mg^{2+} or by chelating intracellular Ca^{2+}, indicating that it is elicited by a rise in intracellular Ca^{2+} (Reid et al., 2002). This effect is similar to that observed in in vivo cold-sensitive neurons, where raised extracellular Ca^{2+} can inhibit the stimulatory effect of menthol (Schafer et al., 1986). These results suggest a simple feedback mechanism in which cooling elicits raised intracellular Ca^{2+} through activation of TRPM8, which then causes Ca^{2+}-dependent adaptation and closing of TRPM8, shifting the activation threshold to a slightly lower

temperature (Reid et al., 2002; Reid, 2005). Thus the threshold of the TRPM8-carried current is dynamically modulated in response to the input stimuli.

1.24.4 Endogenous activators and modulators of TRPM8

There is evidence for TRPM8 protein in the spinal cord (Baccei et al., 2003, Tsuzuki et al., 2004; Results section 4.5.4), and in other non-peripheral tissues such as the prostate (Zhang & Barritt, 2004), where activation by cooling temperatures (<28°C) is not a possibility. This therefore suggests the existence of endogenous chemical agonists of the receptor. Several endogenous agonists of the related TRPV1 receptor have been identified, all of which are arachidonic acid derivatives, for example anandamide and N-oleoyldopamine (Julius & Basbaum, 2001). Similarly, TRPM8 has been shown to be activated by several endogenous lipid-based chemicals. Several phospholipids have been shown to activate TRPM8, although whether their primary function is as activators or as regulatory molecules is unknown. In addition, PKC, NGF and pH have all been shown to regulate the function of TRPM8.

$PI(4,5)P_2$

One endogenous substance which appears to function as a co-activator of TRPM8 is the membrane phospholipid phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$), which has been shown to play a key role in the regulation of TRPM8 function (Liu & Qin, 2005; Rohacs et al., 2005). $PI(4,5)P_2$ can elicit TRPM8 activation at temperatures up to 10°C above the normal activation threshold (Liu & Qin, 2005; Rohacs et al., 2005). In addition, $PI(4,5)P_2$ depletion (produced either by intracellular application of "scavenging" polyvalent cations or by wortmannin inhibition of the phosphoinositide 4-kinases which are involved in $PI(4,5)P_2$ formation) inhibits both cold and menthol activation of TRPM8, suggesting that $PI(4,5)P_2$ is required for the activation of TRPM8 by these stimuli (Rohacs et al., 2005). Given this important facilitatory role of $PI(4,5)P_2$ in TRPM8 channel regulation, it has been suggested that $PI(4,5)P_2$ depletion is linked to channel desensitisation (Rohacs et al., 2005). In line with this, application of exogenous $PI(4,5)P_2$ to excised patches restored TRPM8 currents after desensitisation (Liu & Qin, 2005), and co-expression of the type I phosphatidylinositol-4-phosphate 5-kinase, which catalyses formation of $PI(4,5)P_2$,

in TRPM8 expressing cells, significantly attenuated TRPM8 current desensitisation (Rohacs et al., 2005). Further results suggest that $PI(4,5)P_2$ is hydrolysed following TRPM8 activation, as application of the TRPM8 agonists menthol and icilin induced translocation of fluorescent-protein-tagged PLCδ pleckstrin homology domains constructs from the plasma membrane to the cytosol in a Ca^{2+}-dependent manner (Rohacs et al., 2005), an effect which has been previously shown to reflect $PI(4,5)P_2$ depletion (Varnai & Balla, 1998; Stauffer et al., 1998). Therefore it is suggested that elevated Ca^{2+} due to TRPM8 channel activation activates a Ca^{2+}-sensitive PLC, which causes hydrolysis of $PI(4,5)P_2$, and that this depletion accounts for channel desensitisation (Rohacs et al., 2005), although it is not clear whether $PI(4,5)P_2$ depletion can account for the full extent of channel desensitisation. Additionally, these effects have only been studied in TRPM8-transfected cells, and thus the involvement of this mechanism in native cold-and-menthol-sensitive sensory neurons remains to be investigated.

Lysophospholipids

A recent study suggested that the lysophospholipids lysophosphatidylcholine and lysophosphatidylinositol can activate TRPM8. Application of these lipids activated TRPM8-like currents in excised patches from TRPM8-expressing cells, and inhibitors or antisense-knockdown of the Ca^{2+}-independent phospholipase A_2 enzyme which catalyses the formation of these phospholipids, inhibited menthol-evoked Ca^{2+} increases in TRPM8-expressing cells, suggesting that these phospholipids can facilitate the activation of or directly activate TRPM8 (Vanden Abeele et al., 2006).

PKC

PKC activation has been shown to downregulate TRPM8 function. Menthol-evoked currents in TRPM8-expressing cells and in native sensory neurons are significantly attenuated by PKC activation (Premkumar et al., 2005, Abe et al., 2006). This effect appears to be dependent on dephosphorylation of the TRPM8 receptor produced by PKC-mediated activation of protein phosphatases (including protein phosphatase 1, and possibly protein phosphatase 2A), as it is prevented by inhibitors of PKC or of such phosphatases (Premkumar et al., 2005). PKC-dependent downregulation of TRPM8 can be elicited by application of the pro-algesic agent bradykinin (Abe et al., 2006; Premkumar et al., 2005). Analysis of DRG neurons

showed that 29% of neurons with a TRPM8-like cold-and-menthol-sensitive current responded to bradykinin, and that in these neurons, bradykinin application decreased the temperature activation threshold by ~2.6°C, which was inhibited by coapplication of a PKC inhibitor (Linte et al., 2006). Therefore PKC-mediated downregulation of TRPM8 may have significant effects in vivo. For example, in peripheral inflammation, bradykinin could produce inhibition of TRPM8, and this inhibition of a receptor that normally contributes to cool temperature sensation could potentially contribute to the feeling of heat which accompanies peripheral inflammation.

Linte et al. also showed that 48% of presumed TRPM8-expressing DRG cells were responsive to the inflammatory mediator prostaglandin E_2 (PGE_2), and that PGE_2 decreased the temperature activation threshold of these neurons by ~5.1°C, which was blocked by an inhibitor of PKA (Linte et al., 2006). These results suggest firstly that TRPM8 is functionally modulated by PKA, and secondly that TRPM8 may be doubly inhibited in inflammatory states, by both PGE_2 and bradykinin-mediated effects.

pH

TRPM8 is functionally modulated by pH (Andersson et al., 2004; Behrendt et al., 2004). Acidification of the extracellular solution inhibited the responses of TRPM8-expressing CHO (Chinese hamster ovary) cells to both icilin and cold, as measured in Ca^{2+}-imaging studies (Andersson et al., 2004). The half-maximal inhibitory effect on Ca^{2+} responses evoked by 1 μM icilin or by a 17°C cold stimulus was observed at pH 6.3 (Andersson et al., 2004). In inflamed tissue, extracellular pH levels as low as 5.4 have been recorded (Steen et al., 1992), and therefore the effects observed of extracellular pH on TRPM8 may well be physiologically relevant in peripheral inflammation. Modulation of the intracellular pH via the recording pipette in voltage-clamp experiments also altered icilin-evoked currents through TRPM8, suggesting specifically an intracellular site of action (Andersson et al., 2004). Decreasing the intracellular pH from 7.12 to 6.9 significantly increased the latency of icilin-activated currents and decreased the amplitude, and similarly, lowered the temperature activation threshold of TRPM8 currents. Interestingly, the response to menthol appeared completely unaffected by extracellular or intracellular pH (Andersson et al., 2004). Another study did show that responses to menthol were reduced by lowering

extracellular pH to 6.3 or 6.8, but the responses to menthol were less sensitive to pH changes than responses to icilin (Behrendt et al., 2004). This difference in pH sensitivity suggests a difference between the activation mechanisms of the agonists icilin and menthol, in agreement with other results indicating that icilin-mediated activation is Ca^{2+}-dependent whereas menthol-mediated activation is not (Chuang et al., 2004) and results showing that sensitivity to these ligands is conferred by different residues (Bandell et al., 2006).

NGF

TRPM8 is mainly expressed in trkA positive neurons (Peier et al., 2002b; Kobayashi et al., 2005), and in line with this, TRPM8 function appears to be modulated by NGF levels. In DRG cultures, the fraction of cold-and-menthol-sensitive neurons declines with time in culture, and NGF antagonises this effect (Babes et al., 2004; Reid et al., 2002), suggesting that it maintains TRPM8 expression. NGF also sensitises the response of these neurons, shifting the thresholds of activation to warmer temperatures by approximately 2°C (Babes et al., 2004). This sensitising effect may reflect either an NGF-induced increase in the expression level of TRPM8 in these DRG neurons, or a biochemical modulation of the TRPM8 molecule that facilitates TRPM8 activation, as a result of intracellular signalling pathways that are engaged downstream of NGF-receptor activation, as neurotrophins are known to affect neuronal function by both such mechanisms (Pezet & McMahon, 2006). The effects of NGF on TRPM8 suggest that TRPM8 expression and function could potentially be altered in neuropathic states, (in which afferents experience altered access to neurotrophic factors).

The overall picture emerging of TRPM8 is of a receptor which functions as an integrator of a number of chemical and physical stimuli, rather than a simple detector of cold temperature stimuli. The response properties of endogenous TRPM8 receptors, and therefore the response properties of the relevant subset of cold-sensitive neurons, will be determined not just by the nature of the particular activating stimulus, but will also be affected by the stimulus-history (such as previous exposure to cold or chemical ligands) and the intracellular environment (such as pH, or activation of signalling pathways which modify TRPM8) of the

TRPM8-expressing cells. It is likely that in future, additional activators and modulators of TRPM8 function will be identified.

1.24.5 TRPM8 structure and relationship to function

A high-resolution X-ray crystallographic structure of a TRP channel has not yet been achieved, but in common with other 6 TM domain cation channels, TRP channels are thought to exist as tetramers, forming a central pore with the loop between transmembrane segments 5 and 6 (S5 and S6) acting as the selectivity filter (Kedei et al., 2001; Tsuruda et al., 2006). Figure 1.7 illustrates the general 6TM TRP channel structure.

Analysis of channel conductance properties and kinetics shows that TRPM8, TRPV1, TRPV3, TRPM3 and TRPM4 (and possibly other TRPs) are weakly voltage-dependent (Brauchi et al., 2004; Hui et al., 2006; Voets et al., 2004). They show some similarity to the better-known voltage-gated K^+ channels, which are also members of the 6TM superfamily, and therefore their structure and properties have been compared with the voltage-gated K^+ channels. In Kv1.2, and in the voltage-sensitive TRP channels, voltage-sensitivity is conferred by residues within the S4 domain (Nilius et al., 2005).

Molecular biological studies have also identified sequence motifs which confer ligand sensitivity. Studies of channel mutants have established that residues within the putative TM segment 2 and the part of the C-terminal domain known as the TRP domain, confer menthol sensitivity, and that residues within the same domains also affect sensitivity to icilin (Bandell et al., 2006). Residues within the TRP domain also confer stereoselectivity between different menthol isomers (Bandell et al., 2006). Specific amino acids in the TRP domain have also been shown to be critical for cold sensitivity, for activation by $PI(4,5)P_2$ and for channel gating kinetics (Brauchi et al., 2006; Rohacs et al., 2005). However, the residues conferring menthol sensitivity are different to those conferring cold sensitivity, as mutations which abolish menthol sensitivity do not affect temperature sensitivity (Bandell et al., 2006). Whereas menthol sensitivity is conferred by residues in S2 and part of the C-terminal, the intracellular loop between S2 and S3 is critical for icilin-mediated activation of the channel (Chuang et al., 2004), suggesting fundamental differences

between the mechanisms of icilin and menthol-activation of TRPM8. Interestingly, the chicken orthologue of TRPM8 is insensitive to icilin, because of differences in the coding sequence for this intracellular loop (Chuang et al., 2004), and similarly, differences in the coding sequence of the same region underlie the insensitivity of the chicken version of TRPV1 to capsaicin (Jordt & Julius, 2002).

A coiled-coil structure in the C-terminal domain, which is conserved among all the TRPMs, is necessary for channel assembly and sufficient for formation of the tetramer which constitutes the channel (Erler et al., 2006; Tsuruda et al., 2006). This suggests that there may be a common strategy, among the TRPM family at least, for channel assembly.

1.24.6 TRP channel gating

Analysis of the voltage-sensitivity and channel kinetics of TRPM8 and other temperature-sensitive TRPs has led to a general model for the gating of these temperature-sensitive channels. Voets et al. found a tight link between voltage-dependent activation and temperature-dependence in both TRPM8 and TRPV1, and therefore proposed that temperature changes activate these channels by shifting the voltage-dependent activation curve to physiologically relevant potentials, resulting in activation (Liman, 2006; Nilius et al., 2005; Voets et al., 2004). The effect of ligands such as menthol may therefore be to shift the voltage-activation curve, so that the channel becomes active at higher temperatures (in the case of TRPM8) (Voets et al., 2004). Voets et al. further proposed a thermodynamic and kinetic model for temperature-sensitive channel gating, in which temperature sensitivity of channels will occur whenever there is a significant difference between the activation energies associated with opening and closing of the channel. If the activation energy for opening the channel is much less than that for closing the channel, as for TRPM8, the overall open probability of the channel will increase upon cooling. Conversely, if the activation energy for opening the channel is much greater than that for closing the channel, as for TRPV1, then the open probability of the channel will increase upon heating (Nilius et al., 2005; Voets et al., 2004).

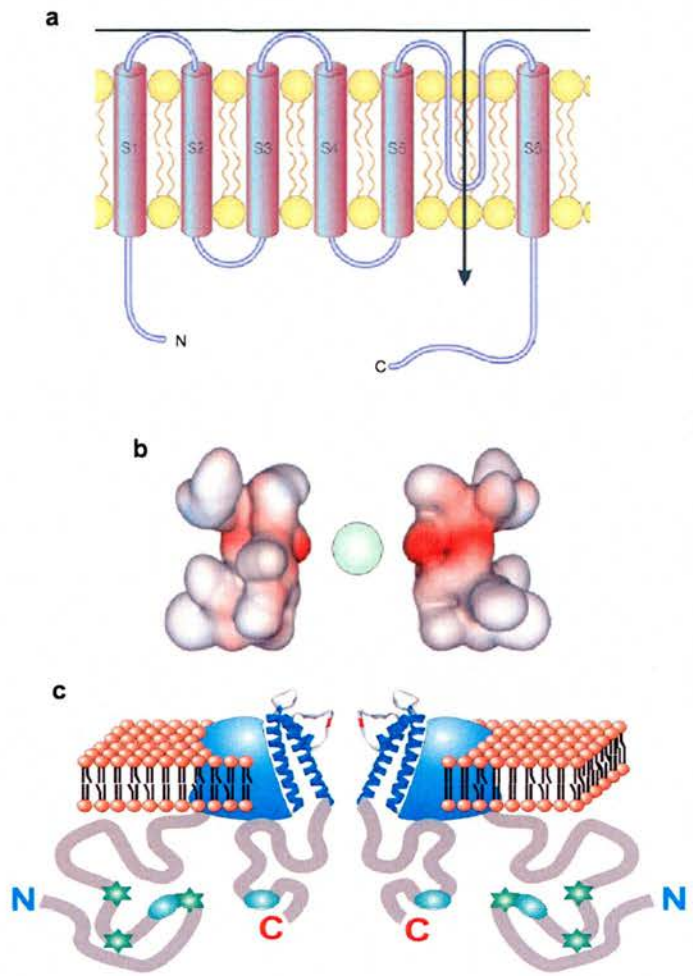


Figure 1.7 TRP channel structure
Figure illustrates:
a) the basic structure of TRP channel subunits, with 6 transmembrane domains (S1 to S6), with the loop between S5 and S6 acting as the selectivity filter.
b) space-filling molecular model of the selectivity filter of the TRP channel pore.
c) schematic to show tetrameric TRP channel structure in the membrane.
All figures kindly provided by Bernd Nilius.

1.24.7 TRPM8 expression

TRPM8 expression is observed in subpopulations of sensory neurons in DRG and trigeminal ganglia (McKemy et al., 2002; Peier et al., 2002b), and in nodose ganglia (Zhang et al., 2004) and dental primary afferent neurons (Park et al., 2006). Nervous system expression appears to be confined to the peripheral nervous system, as Northern blots showed no mRNA expression in spinal cord or brain tissue (McKemy et al., 2002; Peier et al., 2002b). In situ hybridisation studies have shown TRPM8 mRNA in 5 – 10% of DRG neurons, in neurons with diameters consistent with those of C fibre neurons (McKemy et al., 2002; Peier et al., 2002b). Slightly higher levels of TRPM8 expression have been reported by another group – up to 23% of DRG neurons - using high-sensitivity cRNA hybridisation and immunohistochemistry, (Katsura et al., 2006; Kobayashi et al., 2005). This group also observed TRPM8 mRNA in up to 19% of myelinated afferents, which also had small diameters, suggesting Aδ fibres (Kobayashi et al., 2005). TRPM8 mRNA does not appear to be co-expressed with the nociceptor markers CGRP or IB-4 (Peier et al., 2002b). However, TRPM8 is generally (~98%) co-localised with the NGF receptor trkA (Kobayashi et al., 2005; Peier et al., 2002b), which typically marks small-diameter, peptidergic afferents (Averill et al., 1995); although the lack of co-expression with CGRP suggests that TRPM8 may be found in neuronal populations distinct from peptidergic nociceptive afferents. The issue of TRPM8 co-expression with TRPV1 is controversial. Several in situ hybridisation studies have shown no overlap in expression (Kobayashi et al., 2005; Peier et al., 2002b), whereas another group suggested that 29% of TRPM8-expressing cells also expressed TRPV1 (Okazawa et al., 2004). Ca^{2+}-imaging studies have shown up to 50% of menthol-sensitive cells also respond to capsaicin in cell culture (Babes et al., 2004; McKemy et al., 2002; Xing et al., 2006). Some reports have suggested that co-expression of TRPM8 and TRPV1 is an artefact of culture produced by NGF as co-expression of capsaicin and menthol responses was readily detected in cultures with, but not without NGF (Story et al., 2003; McKemy, 2005). However, TRPM8 and TRPV1 mRNA co-expression has been observed in tissue in some reports (Okazawa et al., 2004), and co-expression of capsaicin and menthol responses has been observed within as little as 2 hours after culture, or in cultures without NGF (Babes et al., 2004, Reid, 2005).

There may be a species difference, as co-expression has only been reported in rat tissue (Babes et al., 2004; McKemy et al., 2002; Okazawa et al., 2004), and not in mouse (Peier et al., 2002b) – however, lack of co-expression has also been reported in rat (Kobayashi et al., 2005). Therefore this issue remains unresolved.

The expression pattern of TRPM8 is therefore consistent both with expression in populations of non-nociceptive neurons, (which do not express typical nociceptive markers such as IB-4, CGRP or TRPV1, and which are presumably innocuous cold-sensitive neurons), and with expression in some nociceptive neurons, (due to the data showing co-expression with TRPV1). Analysis of cold-sensitive trigeminal neurons showed that those responding to low-threshold cooling (with thresholds between 30 and 25°C) were much more likely to express TRPM8 mRNA (as shown by single cell RT-PCR) than those with thresholds under 25°C (Nealen et al., 2003), suggesting that TRPM8 may be predominantly expressed by innocuous cold-sensitive afferents. A recent report examined dissociated DRG neurons without culture and identified two classes of menthol-sensitive neurons, one of which had non-nociceptive properties including capsaicin-insensitivity, ATP-insensitivity, and expression of TTX-S Na^+ currents only, and a second population which had nociceptive characteristics including sensitivity to capsaicin and ATP and expression of both TTX-S and TTX-R Na^+ channels (Xing et al., 2006). The non-nociceptive population showed greater sensitivity to menthol: whereas both populations could be activated by 100 μM menthol, three times as many non-nociceptive neurons could be activated by 10 μM menthol as nociceptive neurons, and the non-nociceptive population also responded to 100 μM menthol with larger increases in Ca^{2+}-induced fluorescence or single-cell currents (Xing et al., 2006). This report therefore suggests that TRPM8 may be expressed by both innocuous cold-sensitive neurons and cold-sensitive nociceptors, but is expressed at higher levels in innocuous cold-sensitive neurons, conferring greater sensitivity to TRPM8-activating stimuli on these.

TRPM8 protein is presumed to be on peripheral terminals of sensory neurons, however electrophysiological evidence also suggests a location on central terminals of primary afferents (Baccei et al., 2003; Tsuzuki et al., 2004). Studies of the primary afferent: dorsal horn synapse in spinal cord slices (Baccei et al., 2003) and in DRG afferent: dorsal horn neuron co-cultures (Premkumar et al., 2005; Tsuzuki et al.,

2004) found that TRPM8 activation by menthol increased mEPSC frequency but not amplitude (Baccei et al., 2003; Premkumar et al., 2005; Tsuzuki et al., 2004) suggesting a location at presynaptic afferent terminals in the dorsal horn.

TRPM8 expression outside the nervous system

In addition to a location in sensory nervous tissue, TRPM8 mRNA and protein is also found in prostate tissue (Bidaux et al., 2005; Thebault et al., 2005; Tsavaler et al., 2001; Zhang & Barritt, 2004, 2006) in the bladder (Stein et al., 2004; Tsukimi et al., 2005) and in pulmonary arterial and aortic smooth muscle (Yang et al., 2006). These locations suggest a role beyond sensory thermotransduction, adding to the intriguing diversity of TRP channel functions. In prostate tissue TRPM8 is found both in the plasma and endoplasmic reticular membranes of secretory epithelial cells (Bidaux et al., 2005; Thebault et al., 2005), where it appears to function as an androgen-sensitive Ca^{2+} channel (Thebault et al., 2005; Zhang & Barritt, 2004, 2006,). TRPM8 is upregulated in prostate tumours (Thebault et al., 2005; Zhang & Barritt, 2004), and is found in primary tumours of the breast, lung and skin, where it is not normally expressed at all (Tsavaler et al., 2001), suggesting that it may play a role in tumour development. The TRPM8 agonist menthol can induce apoptosis in cancerous prostate cells (Zhang & Barritt, 2004, 2006), suggesting TRPM8 may play a role in cancer cell survival. In the bladder, TRPM8 is found in the urothelium and nerve fibres, and increased expression is correlated with painful and overactive bladder syndromes in humans (Mukerji et al., 2006).

1.25 TRPA1

A second cold-sensitive TRP receptor is TRPA1 (formerly known as ANKTM1) (Story et al., 2003). TRPA1 is unique among the TRPs and constitutes the sole member of the TRPA family; it is distinguished from other channels by the presence of about 14 ankyrin domains in its N-terminal tail (Clapham et al., 2003; Ramsey et al., 2006).

1.25.1 Activation properties of TRPA1

TRPA1 carries a non-selective cation current. When expressed in heterologous systems, TRPA1 shows a rather variable threshold between 12 - 24°C, with maximal activation at temperatures below 17°C (Story et al., 2003), and therefore is activated by significantly lower temperatures than TRPM8. In addition to cold, TRPA1 is activated by a range of noxious, pungent chemicals. These include cinnamaldehyde (from cinnamon); allyl isothiocyanate (found in mustard oil, horseradish, wasabi and brassicas) and related compounds; allicin (from garlic) and related compounds; carvacrol, eugenol (from cloves); methylsalicylate (wintergreen oil); acrolein (found in tear gas and traffic fumes); tetrahydrocannabinol (the psychoactive component of cannabis) and bradykinin (which appears to activate indirectly, through a PLC-dependent mechanism) (Bandell et al., 2004; Bautista et al., 2005, 2006; Jordt et al., 2004; Macpherson et al., 2005;). Icilin is also a TRPA1 agonist, although shows a much lower potency than at TRPM8 and a delayed (possibly indirect) mode of TRPA1 activation (Story et al., 2003).

There is currently controversy surrounding the role of TRPA1 in cold transduction. Other groups have studied TRPA1 and did not find that this receptor was cold-sensitive (Jordt et al., 2004; Nagata et al., 2005). The generation of knockout mice was expected to resolve the function of TRPA1, but instead has increased confusion surrounding the role of this receptor, as two separate groups found different results. One group found a 50% reduction in withdrawal responses to a 0°C cold-plate test, suggesting TRPA1 is involved in responses to noxious cold (Kwan et al., 2006), but the second group reported normal responses to 0, -5 and -10°C cold-plate tests (Bautista et al., 2006), and thus the involvement of TRPA1 in cold sensitivity remains controversial. However, other results support a role for TRPA1 in noxious cold sensitivity. Genetic analysis of human populations showed that genetic variations in TRPA1 contribute to variations in cold pain sensitivity in humans (Kim et al., 2006), suggesting that TRPA1 is involved in noxious cold sensation. A role for TRPA1 in cold hypersensitivity is suggested, as antisense knockdown produced a decrease in the development of cold allodynia in the SNL model of neuropathy and a model of inflammatory pain (Katsura et al., 2006; Obata et al., 2005). In contrast, there was no such effect of antisense knockdown of TRPM8

(Katsura et al., 2006), suggesting a role for TRPA1, but not TRPM8, in cold allodynia. Therefore while the controversy remains, a number of reports suggest a role for TRPA1 in noxious cold processing.

The role of TRPA1 in response to noxious chemical stimuli is clearer, as many reports demonstrate that TRPA1 is sensitive to a variety of noxious chemicals, and indeed both groups of TRPA1 knockout mice showed deficiencies in licking and flinching behaviour evoked by topical mustard oil (Bautista et al., 2006), intraplantar mustard oil (Kwan et al., 2006) and intraplantar bradykinin (Bautista et al., 2006; Kwan et al., 2006), suggesting that TRPA1 is indeed a key mediator of sensitivity to these noxious stimuli.

TRPA1 is also found in the inner hair cells of the cochlea, and has been proposed to play a role in the mechanosensitive transduction of the inner hair cells (Corey et al., 2004; Nagata et al., 2005). However, TRPA1 knockouts show normal auditory function, suggesting that TRPA1 may not be responsible for inner ear hair cell transduction (Kwan et al., 2006; Bautista et al., 2006). TRPA1 has also been implicated in somatosensory mechanotransduction, as one group of knockout mice showed a decrease in sensitivity to von Frey hair application (Kwan et al., 2006). However, this decrease was slight and the second knockout group showed no such decrease (Bautista et al., 2006), and so further studies are needed to resolve the extent to which TRPA1 may contribute to mechanosensation in vivo.

1.25.2 TRPA1 expression

TRPA1 is expressed in trigeminal, dorsal root and nodose ganglia (Nagata et al., 2005; Story et al., 2003; Zhang et al., 2003). Different studies have shown very different levels of TRPA1 mRNA expression – from 3% (Story et al., 2003), to 32% (Obata et al., 2005) to 55% (Nagata et al., 2005) of DRG neurons. TRPA1 is expressed in small unmyelinated fibres, and co-expression has been observed with CGRP, SP, and TRPV1 (Nagata et al., 2005; Story et al., 2003). These data suggest that TRPA1 is expressed primarily in peptidergic C fibre nociceptors, which are likely to be polymodal, as co-expression of TRPA1 and TRPV1 would confer sensitivity to noxious heat, noxious cold and noxious chemicals. In contrast, TRPA1/TRPM8 co-expression has not been observed (Kobayashi et al., 2005;

Nagata et al., 2005; Story et al., 2003), suggesting that these receptors are expressed by distinct neuronal populations. Northern blots show that TRPA1 mRNA is absent from the CNS, suggesting that its role is confined to the peripheral nervous system (Story et al., 2003).

1.26 Alternative cold-sensitive channels

Additional mechanisms beyond TRPM8 and TRPA1 are implicated in cold transduction (Reid, 2005). As discussed above, the difference in threshold (~6°C) between TRPM8-transfected cells and native TRPM8-like cold-sensitive currents suggests either intrinsic modulation of TRPM8 or the existence of additional cold-sensitive currents in native sensory neurons (Reid, 2005). Furthermore, TRPM8 and TRPA1 expression cannot account for all cold sensitivity in DRG neurons. Populations of cold-sensitive neurons (with thresholds between 30 and 20°C) have been identified that do not respond to either the TRPM8 agonist menthol or to the TRPA1 agonist mustard oil (Babes et al., 2004; Munns et al., 2006), and that do not express TRPM8 or TRPA1, as shown by RT-PCR (Babes et al., 2006). Treatment with the TRPM8 channel blocker BCTC blocks responses to menthol but not all responses to cold in cultured trigeminal neurons and in corneal neuron terminals, suggesting that TRPM8 is involved but cannot does not wholly mediate sensory neuron responses to cold (Madrid et al., 2006; Viana et al., 2006). Sympathetic neurons of the superior cervical ganglia respond to cold but do not generally respond to agonists of either TRPM8 or TRPA1, indicating that alternative mechanisms may mediate cold sensitivity in the sympathetic nervous system (Munns et al., 2006).

Additional cold-sensitive currents and receptors have been identified. These include a hyperpolarising K^+ current which is deactivated by cooling, thus leading to depolarisation (Reid & Flonta, 2001b; Viana et al., 2002). Several 2-pore domain K^+ channels have been identified which are deactivated by decreases in temperature, and could thereby mediate this effect. TREK-1 is activated on warming, with a threshold of ~25°C (Kang et al., 2005) and maximal sensitivity between 32 and 37°C, and is expressed in small and medium-sized DRG neurons (Maingret et al., 2000). However, in argument against a role for TREK-1 in sensory cold transduction,

TREK-1 blockers had no effect on responses of cold-sensitive DRG neurons (Thut et al., 2003), and TREK-1 knockout mice show unaltered cold sensitivity (Alloui et al., 2006). TREK-1 is expressed at high levels in the hypothalamus, and this together with its high sensitivity at temperatures around 37°C suggests that it may be involved in temperature homeostasis (Maingret et al., 2000). TREK-2 (Bang et al., 2000) is also temperature sensitive, with a threshold of around 25°C (Kang et al., 2005). Currents with properties resembling those of TREK-2 have been detected in up to 28% of DRG neurons (Kang et al., 2005). The K^+ channel TRAAK is also deactivated by decreasing temperature, with a threshold of around 31°C, and TRAAK-like currents are observed in around 8% of DRG neurons (Kang et al., 2005). These and potentially other K^+ channels may contribute to cold sensitivity in DRG neurons.

In addition to these K^+ conductances, the epithelial Na^+ channel ENaC has also been demonstrated to respond to cold with an increase in conduction, half-maximal stimulation occurring at 25°C (Askwith et al., 2001). However, as yet little is known about the contribution of this channel to in vivo cold transduction. Inhibition of the Na^+/K^+ ATPase has also been implicated in cold sensitivity, as blockade of this with ouabain facilitates cold receptor firing (Pierau et al., 1974). However, later work suggests that this role is probably minor as ouabain elicited only a minor depolarisation, and no action potentials, in cold-sensitive DRG neurons (Reid & Flonta, 2001b).

Therefore in conclusion it is clear that cold sensory transduction is a complex process, involving multiple receptor mechanisms. Much more work is needed to resolve the contribution of the mechanisms described above to cold transduction in vivo.

1.27 Aims of the project

- To examine the role of the TRPM8 receptor in pain processing. The initial hypothesis was that the TRPM8 receptor could be the molecular mediator of the phenomena of both cooling-induced analgesia and menthol-induced analgesia, and

therefore I examined the effect of TRPM8 activation in models of chronic pain and normal animals.

- To examine the localisation of TRPM8 in afferent neurons and the spinal cord, and any changes in such localisation that occur in chronic pain states.
- To investigate mediators of the effects of TRPM8 activation –I specifically investigated the role of the Group II and Group III mGluRs in mediating effects occurring downstream of TRPM8-expressing neurons.
- To compare the role of TRPM8 with that of the related TRPA1 receptor in pain processing; although TRPA1 was a minor focus of this project.

These aims were addressed experimentally by:

- Topical and intrathecal drug application in models of neuropathic and inflammatory pain and in naïve animals. The effects of TRPM8 and TRPA1 agonists, and agonists and antagonists of various other receptors, principally the Group II and Group III mGluRs were assessed by measurement of behavioural reflex thresholds to sensory stimuli.
- Saphenous nerve recordings to assess the response of primary afferent populations to selective TRPM8 activators.
- In vivo antisense knockdown of TRPM8 followed by behavioural testing, electrophysiology, and protein studies in order to assess the involvement of this receptor in mediating the effects of TRPM8 activators.
- Western blot and immunohistochemical studies of spinal cord and DRG tissue in order to investigate TRPM8 and mGluR localisation and changes in expression in chronic pain models.
- In vivo extracellular recordings of spinal cord neurons to assess the effect of peripheral TRPM8 activation on dorsal horn neuronal processing.

Chapter 2: Materials

2.1 Anaesthetics

- Halothane (Zeneca Ltd., Cheshire, UK)
- Urethane (Sigma Chemical Co., UK)
- α-chloralose (Acros Organics, Geel, Belgium)

2.2 Animal models

- Surgical instruments: scalpel, blunt forceps, blunt scissors, fine curved and straight watchmaker's forceps, needle holders, bonecutters, iris scissors, fine scissors (InterFocus Ltd., Haverhill, UK)
- 25Gx1 needle (Terumo, New Jersey, USA)
- 1 ml syringe (BD Biosciences, Oxford, UK)
- 5.0 coated vicryl sutures (Ethicon Ltd., Edinburgh, UK)
- Lysolecithin (α-lysophosphatidylcholine; Cat. No. L4129, Sigma Chemical Co., UK)
- Chromic cat gut (USP 4/0) (SMI AG, Hunningen, Belgium)
- Freund's complete adjuvant (Cat. No. F5881, Sigma Chemical Co., UK)
- Antisense and mis-sense oligonucleotides (MWG Biotech, Ebersberg, Germany)
- Alzet Minipump, models 2001, 2002 (Charles River Ltd., Margate, UK)
- Vinyl tubing and polyethylene tubing for canulae (Charles River Ltd., Margate, UK)
- 0.9% sterile physiological saline (Sigma Chemical Co., UK)
- Hibitane (5% diluted 1:1000, Zeneca Ltd., Cheshire, UK)

2.3 Behavioural testing

- Hargreaves' thermal stimulator, supplied base and glass table (Linton Instrumentation, Diss, UK)
- Semmes-Weinstein von Frey filaments (Stoelting, Wood Dale, Illinois, USA)
- Clear Perspex cages (made on site)

- Clear Perspex box with raised aluminium floor for cold test (made on site)
- Thermistor (Model 2751-K, Digitron Instrumentation Ltd, Devon, UK)

2.4 Drug administration

- 25Gx1 needle (Terumo, New Jersey, USA)
- 1 ml syringe (BD Biosciences, Oxford, UK)
- Clear plastic tank, 25cm by 25 cm by 20cm for topical application of drug (made on site).
- 0.9% sterile physiological saline (Sigma Chemical Co., UK)
- Dimethylformamide (Sigma Chemical Co., UK)
- Dimethylsulphoxide (Sigma Chemical Co., UK)
- Ethanol 100% (BDH, UK)
- Icilin - 3,4-Dihydro-3-(2-hydroxyphenyl)-6-(3-nitrophenyl)-(1H)-pyrimidin-2-one (Cat. No. 1531, Tocris Cookson, Bristol, UK)
- LY 341495 - (2S)-2-Amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid (Cat. No. 1209, Tocris Cookson, Bristol, UK)
- UBP1112 - α-Methyl-3-methyl-4-phosphonophenylglycine (Cat. No. 1369, Tocris Cookson, Bristol, UK)
- 2R, 4R-APDC - 2R, 4R)-4-aminopyrrolidine-2,4-dicarboxylate (Cat. No. 1208, Tocris Cookson, Bristol, UK)
- ACPT-III - (1R,3R,4S)-1-aminocyclopentane-1,3,4-tricarboxylic acid (Cat. No. 1113, Tocris Cookson, Bristol, UK)
- AP-4 - (L-(1)-2-amino-4-phophonobutyric acid (Cat. No. 0103, Tocris Cookson, Bristol, UK)
- Naloxone - (5α)- 4,5-Epoxy-3,14-dihydro-17-(2-propenyl)morphinan-6-one hydrochloride (Cat. No. 0599, Tocris Cookson, Bristol, UK)
- NMDA - N-methyl-D-aspartate (Cat. No. 0114, Tocris Cookson, Bristol, UK)
- ACPC - 1-aminocyclopropanecarboxylic acid (Cat. No. 07626, Sigma Chemical Co., UK)

- Capsaicin - (E)-N-[(4-Hydroxy-3-methoxyphenyl)methyl]-8-methyl-6-nonenamide (Cat. No. 0462, Tocris Cookson, Bristol, UK)
- Resiniferatoxin - 4-Hydroxy-3-methoxy-[(2S,3aR,3bS,6aR,9aR,9bR,10R,11aR)-3a,3b,6,6a,9a,10,11,11a-octahydro-6a-hydroxy-8,10-dimethyl-11α-(1-methylethenyl)-7-oxo-2-(phenylmethyl)-7H-2,9β-epoxyazuleno[5,4-e]-1,3-benzodioxol-5-yl]benzeneacetate (Cat. No. 1137, Tocris Cookson, Bristol, UK)
- (-)-menthol - (1R, 2S, 5R)5-methyl-2-propan-2-yl-cyclohexan-1-ol (Cat. No. 588733, Sigma Chemical Co., UK)
- Isomenthol - (1S, 2R, 5R)5-methyl-2-propan-2-yl-cyclohexan-1-ol (Cat. No. 58929, Sigma Chemical Co., UK)
- (+)-menthol (1S, 2R, 5S)5-methyl-2-propan-2-yl-cyclohexan-1-ol (Cat. No. 224464, Sigma Chemical Co., UK)
- Cinnamaldehyde - 3-phenyl-2-propenal (Cat. No. W228605, Sigma Chemical Co., UK)
- Ruthenium red - ruthenium (III) chloride oxide ammoniated hydrate (Cat. No. R2751, Sigma-Aldrich Co. Ltd, UK)
- Allicin - 2-propene-1-sulfinothioic acid-S-2-propenyl ester - (Cat. No. A4440, LKT Laboratories Inc., St Paul, MN, USA)
- Diallyl disulphide (Cat. No. D3201, LKT Laboratories Inc., St Paul, MN, USA)
- Prism 4 (GraphPad software Inc.)

2.5 Tissue processing and Western blotting

- Mercaptoethanol - 2-hydroxy-1-ethanethiol (Sigma Chemical Co., UK)
- Sodium dodecyl sulphate (Sigma Chemical Co., UK)
- Tris-hydroxymethylaminoethane (Sigma Chemical Co., UK)
- Urea – diaminoethanal (BDH, UK)
- Thiourea - sulphanylmethanimidamide (Sigma Chemical Co., UK)
- Protease Inhibitor Cocktail III (Cat. No. 539134, Calbiochem, Merck Biosciences Ltd., Nottingham, UK)

- Hand-held glass homogenizer
- Ystral homogeniser (Scientific Industries Intl. UK Ltd., Loughborough, UK)
- NuPage Gel Electrophoresis System (Invitrogen Ltd., Paisley, UK)
- SeeBlue Plus prestained standard molecular weight markers (Cat. No. LC5925, Invitrogen, Ltd., Paisley, UK)
- Glycerol (Sigma Chemical Co., UK)
- Bromophenol blue (Sigma Chemical Co., UK)
- 4-12% Bis-Tris NuPage gels (Cat. No. NP0321, Invitrogen Ltd., Paisley, UK)
- 3-8% Tris-Acetate gels (Cat. No. EA0375, Invitrogen, Ltd., Paisley, UK)
- MOPS running buffer for electrophoresis (Cat. No. NP0001, Invitrogen, Ltd., Paisley, UK)
- Tris-Acetate running buffer for electrophoresis (Cat. No. LA0041, Invitrogen Ltd., Paisley, UK)
- Immobilon- P^{SQ} membrane (Millipore, Watford, UK)
- NuPage Transfer buffer (Cat. No. NP0006, Invitrogen Ltd., Paisley, UK)
- Methanol 100% (Fisher Scientific UK Ltd., Loughborough, UK)
- Ethanoic acid 100% (Fisher Scientific UK Ltd., Loughborough, UK)
- Coomassie Blue (GE Healthcare UK Limited, Bucks, UK)
- Albumin Bovine Serum Fraction V (Sigma Chemical Co., UK)
- 0.1M PBS, pH 7.4
- Tween-20 (Sigma Chemical Co., UK)
- Marvel milk powder
- Enhanced Chemiluminescent Detection Reagents: Lumiglo & hydrogen peroxide, (Cell Signalling, Danvers, Massachusetts, USA)
- Hyperfilm for ECL (GE Healthcare UK Ltd., Bucks)
- Scananalysis programme (Biosoft, Cambridge UK)

2.5.1 Antibodies

- Rabbit polyclonal anti-TRPM8 (residues 278-292 and 1090-1104; human; Cat. No. ab3243, Abcam plc, Cambridge, UK)

- Rabbit polyclonal anti-TRPV1 (Cat. No. AB5889, Chemicon International Ltd., Harrow, UK)
- Rabbit polyclonal anti-mGluR7 (Cat. No. ab07-239, Upstate, Charlottesville, Virginia, USA)
- Rabbit polyclonal anti-mGluR2/3 (Cat. No. AB1553, Chemicon International Ltd., Harrow, UK)
- Rabbit polyclonal anti-mGluR3 (Cat. No. ab10309, Abcam plc, Cambridge, UK)
- Rabbit polyclonal anti-mGluR4 (Cat. No. 51-3100, Zymed, Invitrogen Ltd., UK)
- Mouse monoclonal anti-GAPDH (Cat. No. MAB374, Chemicon International Ltd., Harrow, UK)
- HRP-linked donkey anti-rabbit secondary (Cat. No. AP182P, Chemicon International Ltd., Harrow, UK)
- HRP-linked donkey anti-mouse secondary (Cat. No. AP192P, Chemicon International Ltd., Harrow, UK)

2.5.2 Cell culture and antigen pre-absorption

- Dulbecco's modified Eagle's medium (DMEM; Gibco, Invitrogen, Paisley, UK)
- 10% normal calf serum (Gibco, Invitrogen, Paisley, UK)
- 100 units/ml penicillin (Gibco, Invitrogen, Paisley, UK)
- 100 μg/ml streptomycin (Gibco, Invitrogen, Paisley, UK)
- 20 ml Hank's Buffered Saline Solution (HBSS; Gibco, Invitrogen, Paisley, UK)
- 10 ml Trypsin –EDTA (trypsin 0.5g/l, EDTA 0.2g/l, NaCl 0.85g/l; Gibco, Invitrogen, Paisley, UK)
- 12 well plates (Greiner Bio-0ne, Gloucestershire, UK)
- 2% Ultroser G (USG; Pall Biosciences, Portsmouth, Hampshire, UK)
- Human TRPM8 cDNA clone (Cat. No. TC122185, OriGene Technologies Inc., Rockville, MD, USA)

- pCMV6-XL4 expression plasmid (OriGene Technologies Inc., Rockville, MD, USA)
- GeneJuice Reagent (Novagen, Merck Biosciences Ltd., Nottingham, UK)

2.6 Immunohistochemistry

- OCT embedding compound (Cell Path plc. Powys, Wales, UK)
- Isopentane (BDH, UK)
- Cryostat chucks
- Freezing spray (Greenhill Chemical Products Ltd., Burton-on-Trent, UK)
- Toluidine Blue (Sigma Chemical Co., UK)
- Glass coverslips (22 x 50mm) (Merck-BDH, UK)
- Poly-L-lysine coated glass slides (Merck-BDH, UK)
- Normal goat serum (Vector Laboratories, Burlingame, California, USA)
- Triton X-100 (Sigma Chemical Co., UK)
- Fish skin gelatin (Cat. No. G7765, Sigma Chemical Co., UK)
- Vecta-Shield mounting medium (Vector Laboratories, Burlingame, California, USA)
- ImmEdge hydrophobic barrier marker pen (Vector Laboratories, Burlingame, California, USA)
- 0.1M PBS, pH 7.4

2.6.1 Antibodies and peptides

- Rabbit polyclonal anti-TRPM8, (residues 656-680, rat, Cat. No. H-050-50, Phoenix Peptides, Arizona, USA)
- TRPM8 peptide antigen (residues 656-680, rat, Cat. No. 050-50, Phoenix Peptides, Arizona, USA)
- Mouse monoclonal anti-neurofilament 200kDa, phosphorylated & non-phosphorylated (Cat. No. N0142, Sigma Chemical Co., UK)
- Mouse monoclonal anti-peripherin (Cat. No. MAB1527, Chemicon International Ltd., Harrow, UK)

- Mouse monoclonal anti-neuronal nuclei (Cat. No. MAB377, Chemicon International Ltd., Harrow, UK)
- Rabbit polyclonal anti-mGluR7 (Cat. No. ab13363, Abcam plc, Cambridge, UK)
- AlexaFluor 568 conjugated goat anti-rabbit (Cat. No. A11036, Molecular Probes, Oregon, USA)
- AlexaFluor 488 conjugated goat anti-mouse (Cat. No. A11029, Molecular Probes, Oregon, USA)

2.6.2 Detection and analysis

- To-pro-3-iodide nuclear stain (Cat. No. T3605, Molecular Probes, Oregon, USA)
- Leica TCSNT confocal microscope (Leica Microsystems GMBH, Germany)
- UTHSCSA Image Tool Version 3.0 image analysis software
- Adobe Photoshop Version 7.0

2.7 Electrophysiology

- McLab Chart programme version 3.6 (AD Instruments Ltd., Oxford, UK)
- AC coupled differential amplifier (3160, Digitimer Ltd., Welwyn Garden City, Herts, UK)
- Digitimer (D100, Digitimer Ltd., Welwyn Garden City, Herts, UK)
- Dual beam oscilloscope (D13, Tektronix Inc., Beaverton, Oregon, USA)
- Isolated stimulator (Mark IV, Lectromed Limited, St Quen, Jersey, Channel Islands)
- Agar (Sigma Chemical Co., UK)
- Liquid paraffin
- Motorised rotating brush
- Spike 2 programme (Version 3.2, Cambridge Electronic Design, Cambridge, UK)
- Neurophore BH2 ionophoresis system (Medical systems, Great Neck, New York, USA)

- **2.8 Statistics**
- Sigmastat software version 2.03

Chapter 3: Methods

3.1 Animal models

3.1.1 CCI model of neuropathic pain

The chronic constriction injury (CCI) model of neuropathic pain (Bennett and Xie, 1988) was used. Animals were anaesthetised in a halothane/oxygen mixture (Zeneca, Cheshire, UK), the sciatic nerve was exposed proximal to the trifurcation at mid-thigh level, and four loose ligatures of chromic cat gut (SMI AG, Hunningen, Belgium) were tied around the nerve, as viewed under x 40 microscope, before suturing and recovery. Rats between 180-250 g were preferred as it was observed that these produced a stronger development of neuropathy. Peak reflex sensitisation was observed between days 9 and 16, when further experiments - drug-testing, tissue removal or electrophysiology - were carried out. Development of sensitisation was always measured behaviourally before carrying out further experiments. Animals were monitored for signs of autotomy, which was very rare, and were culled if this developed.

3.1.2 CFA model of inflammatory pain

A model of inflammatory sensitisation was generated by injecting 100 µl Complete Freund's Adjuvant (CFA; Sigma Chemical Co., UK) into the plantar surface of the hindpaw of anaesthetised rats using a microsyringe and 25-gauge needle. The needle was held as close to the skin surface as possible to minimise observed bleeding and avoid systemic effects due to CFA entry into bloodstream. Any further behavioural experiments were carried out at 24 or 48 hours post-injection and tissue removal was carried out at 48 hours. Controls were performed with 0.9% saline injections.

3.1.3 Lysolecithin model of afferent demyelination-induced pain

This model was set up as described by Wallace et al., 2003. Animals were anaesthetised in a halothane/oxygen mixture, the sciatic nerve was exposed proximal to the trifurcation and surrounding fat removed under x 40 microscope. A 5 mm wide

piece of folded parchment paper was carefully inserted under the nerve to prevent spillage of lysolecithin onto surrounding tissue. Lysolecithin (15 mg/ml in sterile 0.9% saline, Sigma, UK) was applied dropwise over a 2-3 mm length of nerve using a 25-gauge needle microsyringe (Terumo) over a period of 10-13 minutes, ensuring that the nerve was kept moist at all times. Excess lysolecithin was removed by applying sterile saline and dabbing on cotton wool before suturing and recovery. Peak reflex sensitisation was observed between days 4 and 10, when further behavioural experiments or tissue removal was carried out.

3.1.4 Targeted knockdown of TRPM8

Knockdown of TRPM8 and controls were carried out by delivering antisense and mis-sense oligonucleotides to TRPM8 into the intrathecal space of rats from osmotic minipumps.

Antisense and mis-sense oligonucleotides were 22-mers with phosphorothioate bonds at the last two positions at 5' and 3' ends (MWG Biotech, Ebersberg, Germany). Antisense extended from base –10 to base +12 relative to the start of the open reading frame for the rat TRPM8 gene: 5'C*T*CGAAGGACATCTTGCCGT*G*G 3', where * represents phosphorothioate linkages. Mis-sense was designed with 4 inversions of C/G or A/T as appropriate at residues 3, 11, 14 and 22, preserving overall G/C content. BLAST searches of both oligonucleotides indicated no significant complementarity to any known gene sequence.

14-day or 7-day osmotic minipumps (Alzet Minipump, models 2001, 2002; Charles River, UK) containing oligonucleotides (0.25 μg/μl in sterile saline) were sewn into the rat and connecting vinyl and polyethylene canulae inserted under the dura of the spinal cord to level L5/6, infusing a dose of 0.5 μl/hour. The minipumps were filled 24 hours prior to insertion and maintained at 37°C until implantation. In some cases a CCI was set up at the same time, in which case 14-day pumps were used so that oligonucleotides were delivered over the same period as that over which sensitisation develops; in non-CCI animals 7-day minipumps were used. At the end of behavioural testing, electrophysiology experiments, or prior to removal of tissue for biochemistry, a laminectomy was performed and the position of the canula

checked. Only results or tissue from animals where the canula was in place and the animal was healthy were taken. The minipumps were weighed before insertion and following removal to check that they had functioned properly and the whole dose had been ejected. Occasional complications of the surgery included motor effects on hindlimbs (rate of 1 in 15) and swelling of the back (1 in 10); these animals were culled and any results discarded.

3.1.5 Dorsal root rhizotomy

A unilateral dorsal root rhizotomy was performed in rats to establish whether TRPM8 expression in spinal cord was pre or postsynaptic. Under deep anaesthesia, a laminectomy was performed to expose the dorsal roots, which were then transected at the lumbar L5/6 level. The wound was sutured and animals recovered for 8 days before tissue removal and processing for western blot or immunohistochemistry. This time period gave sufficient time for loss of presynaptic input while minimising the time of this severe procedure.

3.2 Behavioural testing

Behavioural reflex responses to noxious thermal, punctate mechanical and 4°C cold stimuli were measured. For each test, a minimum of 3 tests was performed to establish a consistent baseline before drug application or to measure development of sensitisation before any other type of experiment. Animals were habituated to testing by performing a series of 3 baseline readings (mechanical and thermal, and cold if that was to be tested) each day for 3 days before performing tests from which results were recorded. Without habituation animals gave erratic baseline results and also showed behaviours that were problematic to the testing procedure (such as attempting to escape). Where a stable baseline was never obtained animals were not used in further testing – this happened in approximately 1 animal in 20.

3.2.1 Thermal sensitivity

Sensitivity to a noxious thermal stimulus was assessed by measuring latency (s) of paw withdrawal in response to an infrared beam (55 °C, Hargreaves' thermal

stimulator, Linton Instrumentation, Diss, UK) directed to the mid-plantar glabrous surface of the hindpaw. The same area of the paw was targeted each time. The withdrawal response latency was recorded to the nearest 0.1s, and only clear-cut paw withdrawal flicks were recorded: disruption of the beam due to locomotion was discounted, and a repeat test carried out. A maximum cut-off value of 20 seconds, and a minimum of 5 minutes recovery time between each test avoided tissue damage and sensitisation (as established by previous experimentation).

3.2.2 Mechanical sensitivity

Sensitivity to mechanical stimuli was assessed by measuring the 50% withdrawal threshold to calibrated Semmes-Weinstein von Frey filaments (Stoelting, Illinois, US). Threshold was recorded as the indentation pressure (mN/mm^2) required to elicit withdrawal to ≥50% of hair applications, from a series of increasing hair pressures (Chaplan et al., 1994). Threshold was recorded as that established by the series of increasing pressures – the response to applying decreasing pressures was not recorded. A minimum of 6 applications of the hair to the foot was used to establish the withdrawal threshold. A maximum hair of pressure 4830.62 mN/mm^2 was set to avoid tissue damage. The hair pressures used and their corresponding forces in grammes are displayed below.

Von Frey hair pressure (mN/mm^2)	Von Frey hair force (g)
318.19	3.63
449.88	5.495
654.18	8.511
848.49	11.749
977.81	15.136
1609.52	28.84
3327.7	75.858
4830.62	125.892

3.2.3 Cold sensitivity

Sensitivity to cold develops de novo in the CCI and lysolecithin models of pain. Responses to cold were measured by the suspended paw elevation test. Animals were placed in a Perspex box with an elevated aluminium floor, containing ice-cold (4 °C) water to a depth sufficient to immerse the hindpaw, but not touch the body of the animal. Rats were allowed 10 s to acclimatise and then the number of seconds the animal raised its hindpaw above the water over a 20s period was recorded as the suspended paw elevation time (SPET). Tests were repeated at 10 minute intervals to allow recovery of hindpaw temperature between tests.

3.3 Drug administration

Drug effects were assessed by behavioural measurements of threshold to noxious and innocuous stimuli. A minimum of three pre-drug tests were performed to produce a mean baseline value. Following drug administration, tests were repeated every 5 minutes for 60-80 minutes for thermal and mechanical stimulation (measured in the same experiment) or every 10 minutes for SPET response. An n of 6 rats was considered sufficient for most drugs. Rats were tested in groups of 3 and thermal and mechanical values were ascertained at the same time – in other words a thermal test was performed followed by a mechanical test, every 5 minutes over the time period.

3.3.1 Intrathecal application of drugs

Drugs were administered into the L5/6 intrathecal space of anaesthetised rats in a volume of 50 µl, using a 1 ml syringe with 25-gauge needle (BD Biosciences, Oxford, UK). Prior experiments with Pontamine Sky Blue dye (Merck-BDH, UK) established the correct site of injection. Testing commenced 15 minutes after injection to allow recovery from anaesthesia: to avoid a false analgesic result and prevent tissue sensitisation caused by delayed responses. Previous experiments by our group and others (Garry et al., 2003a, b Soliman et al., 2005) found complete recovery from anaesthetic by 15 minutes.

The following drugs were applied intrathecally: icilin (2.5-200 µM in saline with 0.2% dimethylformamide, DMF), LY 341495 (10-100 µM in saline), UBP 1112 (10-

200 μM in saline), 2R, 4R-APDC ((2R, 4R)-4-aminopyrrolidine-2,4-dicarboxylate), 300 μM in saline), ACPT-III ((1R,3R,4S)-1-aminocyclopentane-1,3,4-tricarboxylic acid), 3 mM in saline), AP-4, ((L-(1)-2-amino-4-phophonobutyric acid), 3 mM in saline), naloxone (0.5 mM in saline), NMDA (75, 600 μM in saline) and ACPC (1-aminocyclopropanecarboxylic acid, 15, 120 μM in saline), capsaicin (2-5 mM in saline), resiniferatoxin (10 μM in saline) (all from Tocris Cookson, Bristol, UK), (-)-menthol (4 mM in saline), cinnamaldehyde (0.5, 1.5 mM in saline) and ruthenium red (5 μM in saline) (all from Sigma-Aldrich Chemical Co. Ltd, UK) and allicin (0.5 mM in saline with 0.5% DMF) and diallyl disulphide (DADS, 1 mM in saline with 0.5% DMF) (LKT Laboratories Inc., St Paul, MN, USA). Appropriate vehicles based on 0.9% saline were also tested as controls.

The effect of icilin on thermal and mechanical sensitivity was assessed in naïve animals and CCI, lysolecithin and CFA rats at the peak of behavioural reflex sensitisation (assessed by baseline measurement immediately prior to injection). The effect of icilin on cold hypersensitivity was measured in lysolecithin animals. Responses to icilin at four different doses (0.125 nmol, 0.5 nmol, 2.5 nmol and 10 nmol) were assessed in CCI animals. Dose-response effects were analysed with Prism 4 (GraphPad Software Inc.). The effect of the TRPM8 agonist (-)-menthol was assessed in CCI animals. The effects of icilin co-administered with the μ-opioid receptor antagonist naloxone, the group II mGluR antagonist LY 341495, the Group III mGluR antagonist UBP 1112, or these antagonists alone were tested in CCI animals. The effects of LY 341495 and UBP1112 were also tested in naïve animals. The effects of the Group II mGluR agonist 2R,4R-APDC and the Group III mGluR agonists AP-4 and ACPT-III were assessed in naïve, CCI and CFA-injected animals. The effects of the TRPA1 agonist cinnamaldehyde alone or together with icilin were assessed in naive and CCI animals. The effects of NMDA, NMDA with the glycine site agonist ACPC, and the NMDA/ACPC combination with icilin were assessed in naïve and CCI animals. The effects of the TRPV1 agonists capsaicin and resiniferatoxin were assessed in naïve and CCI animals but not pursued. Intrathecal icilin (10 nmol) was also tested in the formalin model.

3.3.2 Topical application of drugs

The effects of topically administered drugs were investigated by applying drug solution to the hindpaws for a 5 minute period, followed by sensory testing for 60-80 mins. This was accomplished either by placing rats in a 1 cm deep waterbath containing drug solution, or by very lightly anaesthetising rats and immersing hindpaws in small tubes (modified 15 ml centrifuge tubes) containing 5 ml volume of drug solution. Drug solution was maintained at 30°C to avoid temperature effects. The waterbath was designed to allow the rat to be placed in a shallow volume of drug, such that a standard volume of 400 ml covered the hindpaws but did not wet the body of the rat, and to give sufficient space for the rat to move reasonably freely. Topical application by immersing hindpaws in small tubes was carried out when higher concentration of drug made a 400 ml volume prohibitively expensive. Anaesthetic was maintained at the minimum required and control experiments with vehicle demonstrated that anaesthetic was not sufficient to affect sensitivity. Following removal from the waterbath the rats were rinsed and loosely dried which should have removed most of the drug. No licking or washing behaviour (beyond what is normally observed) was observed following drug administration.

Sensory testing commenced 5 minutes after waterbath application, or 15 minutes following application if anaesthetic was administered. The effects of icilin (2.5 – 500 μM in water with 0.2% DMF) administered as a waterbath were investigated in CCI and naïve animals. The effects of higher doses of icilin (1 mM-5 mM in vehicle of 45% DMF in 0.2% aqueous Tween 20) were measured following administration in small foot-tubes. The effects of topical footbath (-)-menthol (4 mM in 25-80% ethanol) and its stereoisomers isomenthol and (+)-menthol (4-16 mM in 25-80% ethanol) were assessed in CCI and naïve animals. The effects of cinnamaldehyde (1.5 mM in water, as footbath), and cinnamaldehyde with icilin (80 μM in 0.2% DMF) were assessed in CCI and naïve animals. The effects of icilin (80 μM as footbath) were further assessed in CCI animals that had undergone antisense and mis-sense treatment for knockdown of TRPM8, and animals which had recovered from this treatment. The effects of icilin applied immediately following intrathecal injection with either LY 341495 or with UBP 1112 were also investigated in CCI animals. All appropriate vehicles were tested.

The effects of paw immersion at different temperatures (10-22°C water for 5 min) on CCI rats were assessed by mechanical testing only. Testing was carried out immediately after removal of animal from the waterbath, and repeated every minute for 5 minutes. Actual skin temperatures were measured by subcutaneous thermistor probe in an anaesthetised rat and were found to equilibrate to around 0.5°C above bath temperature. The effect of a 16°C immersion on CCI rats following intrathecal administration of LY 341495 or UBP 1112 was also measured.

3.4 Western blot analysis of proteins

3.4.1 Tissue removal and processing for Western blot

DRG and spinal cord tissue from L4-L6 was collected from live animals under deep anaesthesia. A laminectomy was performed to expose the spinal cord, and tissue was dissected out. Spinal cord from injury models was hemisected into ipsilateral and contralateral sections. Spinal cord tissue samples were kept on ice before processing as quickly as possible. To minimise degradation of DRG tissue, DRG were collected onto ice-cold foil, weighed, and homogenised directly.

For preparations of whole lysates, tissue was homogenised in 20 volumes of Laemmli lysis buffer (85% Tris buffer (tris-hydroxymethylaminoethane, 50 mM, pH 7.4, Sigma Chemical Co., UK), 5% mercaptoethanol (Sigma Chemical Co., UK), and 2% sodium dodecyl sulphate (SDS, Sigma Chemical Co., UK)), heated to 70°C for 5 minutes, cooled, aliquoted and stored at –40°C. In some conditions samples were treated with Laemmli buffer containing 6 M urea and 2 M thiourea.

For preparations of crude particulate fractions, spinal cord or DRG tissue was homogenised using a Y-stral homogeniser in 20 volumes of ice-cold Tris buffer (50 mM, pH 7.4, Sigma Chemical Co., UK) containing 1% protease inhibitor cocktail III (Calbiochem, Merck Biosciences Ltd., Nottingham, UK) and spun at 2000 rpm for 5 minutes at 4°C to remove cell debris. The supernatant was decanted and re-centrifuged at 11000 rpm for 45 minutes at 4°C. The supernatant cytosolic fraction was decanted, and 15% of this volume of SDS/mercaptoethanol 2:1 mixture was added. The crude particulate fraction pellet was re-suspended in 1/10 of the original fraction volume of Laemmli lysis buffer.

Tissue from CCI, CFA, naïve, mis-sense and antisense oligonucleotide-treated, and lysolecithin-treated animals was processed.

3.4.2 Western blot procedure

Western blotting was used to detect changes in protein levels in DRG or spinal cord tissue from pain models and naïve models.

Proteins were separated by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) using the NuPage XCell SureLock^{TM} Minicell gel electrophoresis system (Invitrogen, Paisley, UK). Samples (5-8 µl) were mixed with 1 µl loading buffer (0.04%w/v bromophenol blue in glycerol) and loaded into wells on either 3 - 8% Tris-Acetate or 4 - 12% Bis-Tris NuPage gels (Invitrogen, Paisley, UK). Pre-stained standard molecular weight proteins (SeeBlue Plus, Invitrogen, Paisley, UK) were run alongside as a guide. Samples were electrophoresed using appropriate running buffers (Tris-Acetate buffer or MOPS running buffer, NuPage, Invitrogen, UK) and a 200 V potential. Proteins were transferred to a polyvinylidene difluoride Immobilon-P^{SQ} membrane (Millipore, Watford, UK) at 30 V in transfer buffer (5% NuPage transfer buffer, Invitrogen, UK, 10% methanol). Efficacy of transfer and protein loading was assessed by staining membranes with Coomassie blue (0.1% in 30% methanol, 10% ethanoic acid, GE Healthcare Ltd., UK) and destaining in 30% methanol/10% ethanoic acid.

Membranes were incubated in 5% Marvel in phosphate-buffered saline (PBS; 0.1 M, pH 7.4) to block non-specific activity either for 2 hours at room temperature or overnight at 4 °C. Membranes were then washed and incubated with rabbit polyclonal antibodies specific to TRPM8 (1:1000 – 1:100; Abcam plc, Cambridge, UK), TRPV1 (1:100; Chemicon International Ltd, Harrow, UK), TRPA1 (1:50; Abcam), mGluR7 (1:100; Upstate), mGluR2/3 (1:500; Chemicon), mGluR4 (1:60; Zymed), mGluR3 (1:500; Abcam) or a mouse monoclonal antibody to glyceraldehyde -3-phosphate dehydrogenase (GAPDH, 1:750; Chemicon), either for 90 minutes at room temperature or overnight at 4°C. Membranes were washed and incubated with peroxidase-linked secondary antibodies: goat anti-rabbit (1:5000 – 1:2500; Chemicon) or goat anti-mouse (1:10000; Chemicon) for 1 hour at room temperature. Antibodies were made up in 2% bovine serum albumin (BSA) in 0.1M

PBS containing 0.1% Tween-20. Staining was detected by peroxide-linked chemiluminescent substrate and film development (ECL; Cell Signalling, MA, USA, hyperfilm; GE Healthcare UK Ltd., Bucks, UK). Densitometry analysis of protein bands to quantify staining was performed using the ScanAnalysis program (Biosoft, Cambridge, UK). Note: Where two concentrations of antibody are listed, this was due to differences in strength between two different batches supplied. Antibody conditions were optimised for each antibody and for each batch.

3.4.3 Preparation of cell membranes for antigen pre-absorption controls

To identify whether the anti-TRPM8 antibody was indeed specific to TRPM8 a pre-absorption control was carried out by incubating antibody with membranes taken from TRPM8-transfected cells, prior to use on immunoblots.

3.4.3.1 Cell culture

COS 7 cells were continuously cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Invitrogen, Paisley, UK), supplemented with 10% normal calf serum (Gibco), 100 units/ml penicillin (Gibco) and 100 μg/ml streptomycin (Gibco) and kept at 37°C in a 5% CO_2, 95% O_2 humidified environment.

In order to passage or plate out cells, 20 ml Hank's Buffered Saline Solution (HBSS) (Gibco) was applied to the cell surface and left for 20 secs. The HBSS was then removed and the cells were exposed to 10 ml Trypsin –EDTA (trypsin 0.5g/l, EDTA 0.2g/l, NaCl 0.85g/l (Gibco). After one minute this was aspirated off and the cells were incubated at 37°C for 5 minutes. Following incubation, the cells were resuspended in the appropriate medium (as above) before being passaged into 75 cm^2 flasks or 12 well plates (Greiner Bio-0ne, Gloucestershire, UK).

3.4.3.2 Cell transfection

Cells were grown to 60% confluence, then transfected with human TRPM8 cDNA in pCMV6-XL4 expression plasmid or empty vector (both OriGene Technologies Inc., Rockville, MD, USA), using GeneJuice Transfection Reagent (Novagen, Merck Biosciences, Nottingham, UK), according to the manufacturer's instructions. GeneJuice reagent was added to the cDNA at a 3:1 ratio for COS 7 cells, and left to incubate for approximately 15 mins. The medium was removed from the cells and

replaced with 2% Ultroser G (USG; Pall Biosciences, Portsmouth, Hampshire, UK) DMEM. The Gene Juice-cDNA complexes were added to the cells in appropriate quantities. Cells were then grown for 48 hours at 37°C in a 5% CO_2, 95% O_2 humidified environment.

3.4.3.4 Preparation of membranes

Cells were scraped into ice-cold 0.1M PBS containing 1% protease inhibitor cocktail III (Calbiochem, Merck Biosciences Ltd., Nottingham, UK) and homogenised using Ystral homogeniser before centrifuging at 1000 g for 10 min. The supernatant was then centrifuged at 48,000 g for 30 min and the pellet from this was resuspended in antibody buffer.

Rabbit polyclonal anti-TRPM8 antibody (Abcam plc, Cambridge) antibody was added to TRPM8-expressing or control membrane suspensions and rolled at 4°C for 16 h prior to use.

3.5 Immunohistochemistry analysis of protein localisation

3.5.1 Tissue processing for immunohistochemistry

As for Western blot, a laminectomy was performed to expose the spinal cord, and DRG and spinal cord tissue from L4-L6 were dissected out. Tissue was collected in OCT compound (Cell Path plc. Powys, Wales, UK) and fast-frozen in isopentane suspended in liquid nitrogen or dry ice before storing at –80°C prior to cryostat sectioning.

3.5.2 Immunohistochemistry procedure

Immunohistochemistry was used to compare protein levels in nerve-injured and naïve animals, and also to examine co-localisation of particular proteins.

15 μm tissue sections were cut on a cryostat at temperatures of -20°C for DRG and -14°C for spinal cord, and thaw-mounted on poly-L-lysine slides (Merck-BDH, UK). Slides were encircled with hydrophobic barrier pen (ImmEdge, Vector Laboratories, CA, USA). Slides were blocked for 1 hour at room temperature in block containing: 10% normal goat serum (Vector Laboratories, CA, USA), 4% fish skin gelatin (Sigma Chemical Co., UK), 0.2% Triton X-100 (Sigma Chemical Co., UK) and 0.1 M PBS. Slides were incubated overnight with appropriate primary antibodies in

buffer (4% normal goat serum, 4% fish skin gelatin, 0.2% Triton X-100, 0.1 M PBS). Antibodies used were: rabbit polyclonal anti-TRPM8 (Phoenix Peptides, 1:450), mouse monoclonal anti-NF-200 (Sigma, 1:1000), mouse monoclonal anti-peripherin (Chemicon, 1:250), guinea-pig anti-TRPV1 (Chemicon, 1:50), rabbit anti-mGluR7 (Abcam plc, 1:50). Slides were washed, incubated with appropriate secondary antibodies: goat anti-rabbit AlexaFluor 568 1:750, goat anti-mouse AlexaFluor 488, 1:500, or goat anti-guinea-pig AlexaFluor 647 1:500 (all Molecular Probes, OR, USA) in the same buffer excluding Triton X-100. Nuclei were stained with To-Pro-3-iodide nuclear stain (1:1000, Molecular Probes, OR, USA). Slides were coverslipped using Vectashield (Vector Laboratories, USA) and sealed with nail varnish. Concentrations and washing conditions were optimised for each antibody.

Sections were visualised under a Leica TCSNT confocal microscope (Leica Microsystems GMBH, Germany). Image analysis was performed with Image Tool software (UTHSCSA Image Tool Version 3.0) and images prepared using Adobe Photoshop. Cell counts were performed on five to eight randomly selected DRG sections (separation of 100 μm) from each of three animals in each experimental group being analysed, and only neurons with clear nuclei were counted. Fluorescence intensity in spinal cord sections was assessed with Image Tool software and compared between ipsilateral and contralateral dorsal horns, from at least 5 sections from each of three animals in each experimental group to give a rough estimate of ipsilateral-contralateral differences.

3.5.3 Antigen pre-absorption control

Specificity of the antibody against TRPM8 (Phoenix Peptides, CA, USA) was assessed by pre-incubating with the peptide (656-680; Phoenix Peptides, CA, USA), which was dissolved at a stock concentration of 1 mg/180 μl in PBS, and used at a ratio of 5 μg peptide:1 μg antibody, by rolling at 4°C for 16 hours. Control aliquots were treated with PBS alone.

3.6 Electrophysiological analysis

3.6.1 Peripheral recordings of saphenous nerve afferents

Recordings of saphenous nerve were made in naïve animals (n=7) and from animals treated with antisense or mis-sense oligonucleotides to TRPM8 over a period of 4-5 days. Rats were anaesthetised with 25% urethane intraperitoneally (0.6ml/100g) and the saphenous nerve was exposed and dissected from its associated vein and artery. Further dissection under liquid paraffin enabled the identification of afferent preparations comprising a small number of units. The dissected afferents were placed over a bipolar recording electrode and the whole nerve preparation was submerged in liquid paraffin. The hindpaw of the treated animal was secured to a footplate using plaster of Paris coated gauze and the receptive field of the afferent preparation was identified by the induction of impulse activity via gentle brushing of the hindpaw. The electrophysiology apparatus was set up as follows: afferent activity, detected by the bipolar recording electrodes, was amplified using an AC coupled differential amplifier (3160, Digitimer Ltd.) with a band width setting of 80 Hz to 2.5 kHz. Shapes and latencies of individual impulses were identified using the storage facility on the D13 oscilloscope (Tektronix Inc.) and single frame or overlapping multiple frames converted to TTL (transistor-transistor logic) pulses using a spike processor (D130, Digitimer Ltd.) which was connected to a second oscilloscope with audio display and to the MacLab Chart programme (version 3.2). Conduction velocities were measured using electrical excitation of receptive fields with a bipolar needle stimulating electrode. A Digitimer D100 (Digitimer Ltd.) triggered an isolated stimulator (Mark IV, Lectromed Ltd.) to deliver square-wave pulses (0.5-1.0 milliseconds, 0.1-100 V) at levels selected to be just above threshold for few-unit preparations via the stimulating electrodes. The conduction velocity of single identified afferent fibres was determined using bipolar electrodes and the peripheral stimulus technique (Iggo, 1958). An electrical stimulus was delivered to the peripheral receptive field and any resulting action potential discharge was recorded from the nerve preparation. The distance between stimulating and recording electrodes was measured and the time taken for a spike to reach the recording electrode was measured by counting the time between the stimulating potential and

peak action potential on the oscilloscope. Following isolation of few-unit preparations, icilin (200 μM in 0.2% DMF), (-)-menthol (4 mM in 25% ethanol), resiniferatoxin (1 mM in ethanol) or vehicles alone were applied to the hindlimb receptive fields and neuronal responses recorded using the Chart programme. The firing of individual neurons was counted by reading from the Chart recording over a 30 second time-period, and thus the firing rate was calculated. One reading was taken from the background (pre-drug) firing. Three readings were taken following drug application, at what was deemed to be the peak period of firing (or from equivalent times in animals which did not respond to the drug) and the mean calculated, and three readings were taken from the recovery period and the mean calculated.

3.6.2 Central recordings of single dorsal horn neurons

Recordings of spinal dorsal horn neurons were made in CCI animals. Following initial induction with halothane, the jugular vein and trachea were canulated and intravenous anaesthetic delivered: α-chloralose (0.6 mg/kg, Acros Organics Ltd., Belgium) and urethane (1.2 mg/kg, Sigma Chemical Co., UK), with supplementary doses of chloralose (10 mg/ml) throughout the experiment as required. Core body temperature was maintained at 37-38°C by means of a thermostatically controlled heated blanket. The animal was placed in a stereotaxic frame, and the thoracolumbar spinal column was supported using three pairs of swan-necked clamps. A laminectomy was performed at L2-L5, and agar (2% in saline at 37°C) was delivered over the exposed cord to increase mechanical stability. Above the recording region the agar and spinal cord dura were removed and liquid paraffin poured over. Extracellular recordings were made from single multireceptive neurons in laminae I-IV through the centre barrel of a seven- barrelled glass microelectrode filled with 4 M saline (tip-diameter 4-5 μM, DC resistance 5-8 MΩ). Recordings were made at depths of 0 – 1000μm from the spinal cord surface as monitored by the microdrive reading. The output from the electrode was filtered using a pre-amplifier and magnified x1000 using a second pre-amplifier. The output from the second pre-amplifier passed both to an oscilloscope (Tektronix), allowing monitoring of cellular activity, and to a spike processor (Digitimer 130) which digitised the signal. Spike processor output was sent via a 1401 converter to a computer, where it was recorded

and analysed using the Spike2 programme (version 3.2, CED, UK). Sustained firing was recorded as the number of spikes fired integrated over 1 s bins, plotted against time. The receptive fields of hair-follicle innervated neurons on distal hindlimb were identified by an innocuous brush stimulus and multireceptivity established by response to noxious pinch. A motorised rotating brush applied to the receptive field was then used to continuously stimulate neuronal firing. Icilin (200 μM in water with 0.2% DMF) or vehicle (0.2% DMF) was then applied peripherally (via a drug-soaked gauze patch) to part of the receptive field. This part of the receptive field was immediately adjacent to the rotating brush stimulus - obviously it was impossible to simultaneously stimulate with rotating brush and apply drug to the same area, so the drug was applied to the immediately adjacent area. The effects on neuronal responses were recorded and analysed using the Spike2 programme (Version 3.2, CED, UK). The mGluR antagonist UBP 1112 (200 μM in water, pH 8.5), and control saline (1 M saline, pH 8.5) were ionophoresed from the side barrels of the electrode using currents of between 20 nA and 80 nA (Neurophore BH2 ionophoresis system, Medical Systems, Great Neck, NY) to measure effects on neuronal response to icilin.

3.7 Statistics

Data was analysed for statistical significance using Sigmastat software (version 2.03). All data is stated as mean ± standard error of the mean (SEM) and p values <0.05 considered significant. Parametric statistical tests were used to analyse data generated from the Hargreaves' thermal test. Differences in thermal sensitivity between the paw ipsilateral to nerve injury and the contralateral paw were analysed using Student's t-test. Effect of drug treatment was analysed by One-Way ANOVA followed by Dunnett's post-hoc multiple comparisons test. The equivalent non-parametric tests for mechanical sensitivity were Wilcoxon rank test for ipsilateral: contralateral differences, and Friedman Repeated Measures ANOVA followed by Dunn's test for drug effects. Western blot densitometric values were compared with the Wilcoxon test, immunohistochemistry cell counts were analysed by One-Way ANOVA, and electrophysiological spike frequencies analysed by One-Way ANOVA on Ranks.

Chapter 4: Results

4.1 The development of sensitisation in models of chronic pain

Sensitivity of behavioural reflexes to noxious thermal, graded mechanical and cold stimuli was measured to assess the degree of sensitisation in animal models of chronic pain. The models used were the chronic constriction injury (CCI) model of neuropathic pain, the lysolecithin model of demyelination-induced pain, and a model of chronic inflammatory pain generated by injecting Complete Freund's Adjuvant (CFA) into the hindpaw; the timecourses of behavioural sensitisation in these models are shown in Figure 4.1.

Figure 4.1a-c shows the timecourse of behavioural sensitisation following CCI. Significant sensitisation of ipsilateral paw responses to thermal and mechanical stimuli, and de novo development of withdrawal reflexes to 4°C cold were reliably induced following CCI, with no effect on contralateral paw values. Sensitivity peaked between days 9 to 17, and further experiments (such as drug testing, biochemistry or electrophysiological recordings) were carried out in this period.

Figure 4.1d-f shows the timecourse of behavioural sensitisation in the lysolecithin model. As with the CCI model, there was sensitisation of ipsilateral paw responses to thermal and mechanical stimuli, and de novo development of withdrawal reflexes to 4°C cold, with no effect on contralateral paw values. Sensitivity peaked between days 4 to 10, when further experiments were carried out.

Figure 4.1g-i shows the timecourse of behavioural sensitisation following injection of CFA into one hindpaw. The ipsilateral paw showed significant sensitisation of responses to thermal and mechanical stimuli, as was expected, which peaked between 24 and 48 hours. There was also a small trend towards sensitisation of contralateral thermal responses, which was significant at some timepoints, and a slight, non-significant lowering of contralateral mechanical threshold. To further investigate these effects, saline injections were carried out as a further control, and these had no discernible effect on behavioural thresholds. Therefore the small sensitisation of contralateral thermal responses observed in this study following CFA appears to represent a small, but significant effect of CFA treatment. This could be

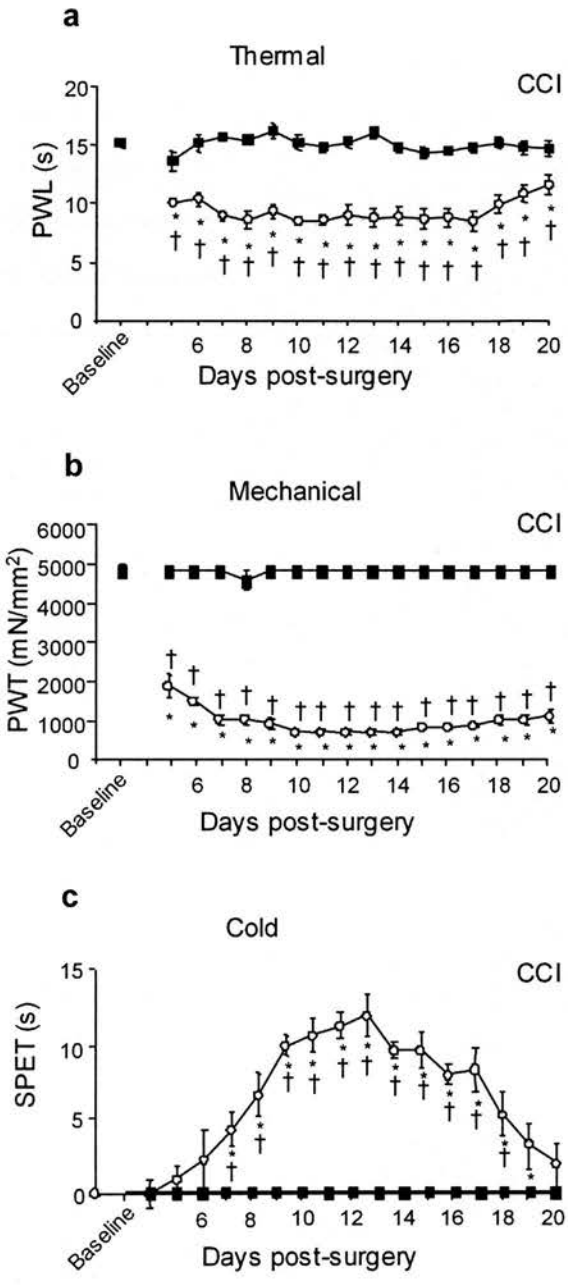
due to CFA entering the bloodstream following injection and becoming systemic. Alternatively, it could represent a contralateral effect of the ipsilateral treatment. Contralateral sensitisation has been observed in other pain models such as the PNL (Seltzer et al., 1990), and SNI (Erichsen & Blackburn-Munro, 2002) models of neuropathic pain. These effects are specific to the area of sciatic innervation, suggesting that the contralateral effects are not mediated by humoural mechanisms but must be mediated by a more specific mechanism. Dorsal horn commissural neurons connect homologous regions of the spinal cord (Szentagothai, 1964), and electrical stimulation of primary afferents terminating in the superficial dorsal horn can affect the responses of contralateral dorsal horn cells, suggesting that commissural connections do indeed have a functional role (Fitzgerald, 1983), and could thereby mediate contralateral changes pain models. The effect observed in the CFA model could reflect these connections between different sides of the spinal cord.

Figure 4.1 Timecourse of sensitisation in models of chronic pain

a, b, c) Timecourse of sensitisation following CCI. Behavioural data show (a): paw withdrawal latency (PWL; s) to a noxious thermal stimulus, (b): paw withdrawal threshold (PWT; mN/mm^2) to graded mechanical stimuli and (c): suspended paw elevation time (SPET; s) for a 20 s exposure to 4 °C water. Data show pre-surgery baselines and measurements taken up to 20 days following CCI, mean ± SEM, n=6 animals. Ipsilateral paw (○) values show development of behavioural sensitisation with little effect on contralateral (■) paw values.

* indicates significant ($p<0.05$) ipsilateral-contralateral differences, as measured by Student's t-test for thermal data, and Wilcoxon test for mechanical/SPET data;

† indicates significant ($p<0.05$) difference from pre-surgery baseline values, as measured by One-Way RM ANOVA followed by Dunnett's post-hoc test for thermal data, and Friedman ANOVA on Ranks followed by Dunn's post-hoc test for mechanical/SPET data.


a
Thermal
CCI
PWL (s)
20
15
10
5
0
Baseline
6
8
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12
14
16
18
20
Days post-surgery
b
Mechanical
CCI
PWT (mN/mm²)
6000
5000
4000
3000
2000
1000
0
Baseline
6
8
10
12
14
16
18
20
Days post-surgery
c
Cold
CCI
SPET (s)
15
10
5
0
Baseline
6
8
10
12
14
16
18
20
Days post-surgery

Figure 4.1 continued:

d, e, f) Timecourse of sensitisation following lysolecithin application to the sciatic nerve. Behavioural data show (d): paw withdrawal latency (PWL; s) to a noxious thermal stimulus, (e): paw withdrawal threshold (PWT; mN/mm^2) to graded mechanical stimuli and (f): suspended paw elevation time (SPET; s) for a 20 s exposure to 4 °C water. Data show pre-surgery baselines and measurements taken up to 15 days following lysolecithin treatment, mean ± SEM, n=6 animals. Ipsilateral paw (○) values show development of behavioural sensitisation with no consistent on contralateral (■) paw values.

* indicates significant ($p<0.05$) ipsilateral-contralateral differences, as measured by Student's t-test for thermal data, and Wilcoxon test for mechanical/SPET data;

† indicates significant ($p<0.05$) difference from pre-surgery baseline values, as measured by One-Way RM ANOVA followed by Dunnett's post-hoc test for thermal data, and Friedman ANOVA on Ranks followed by Dunn's post-hoc test for mechanical/SPET data.

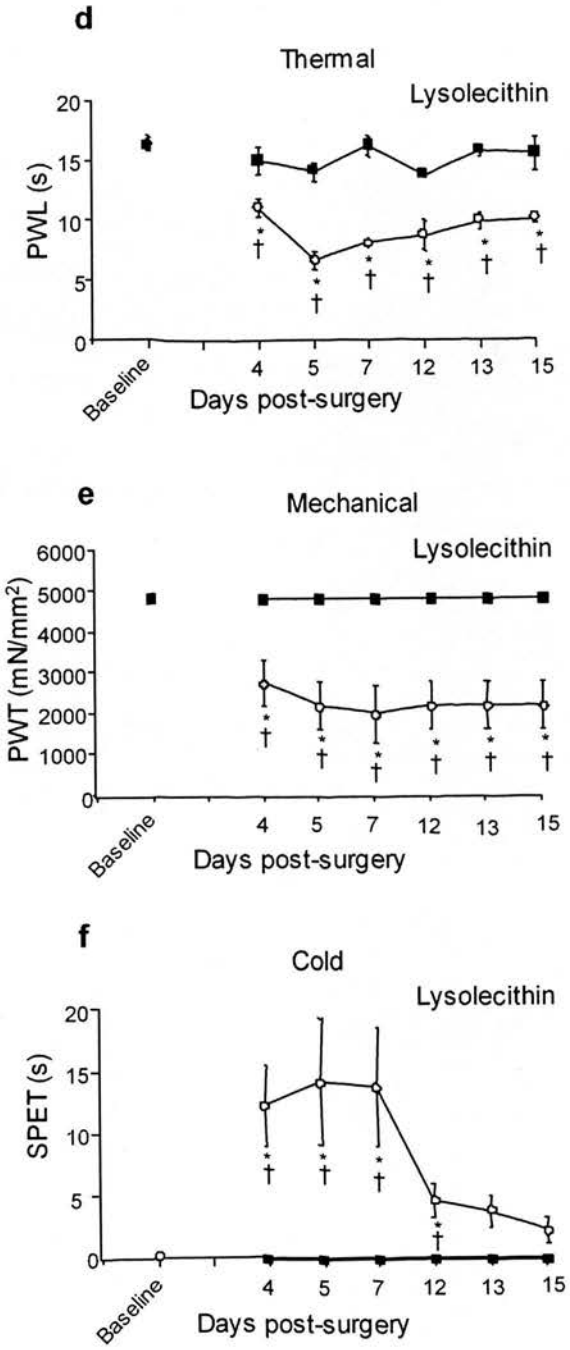
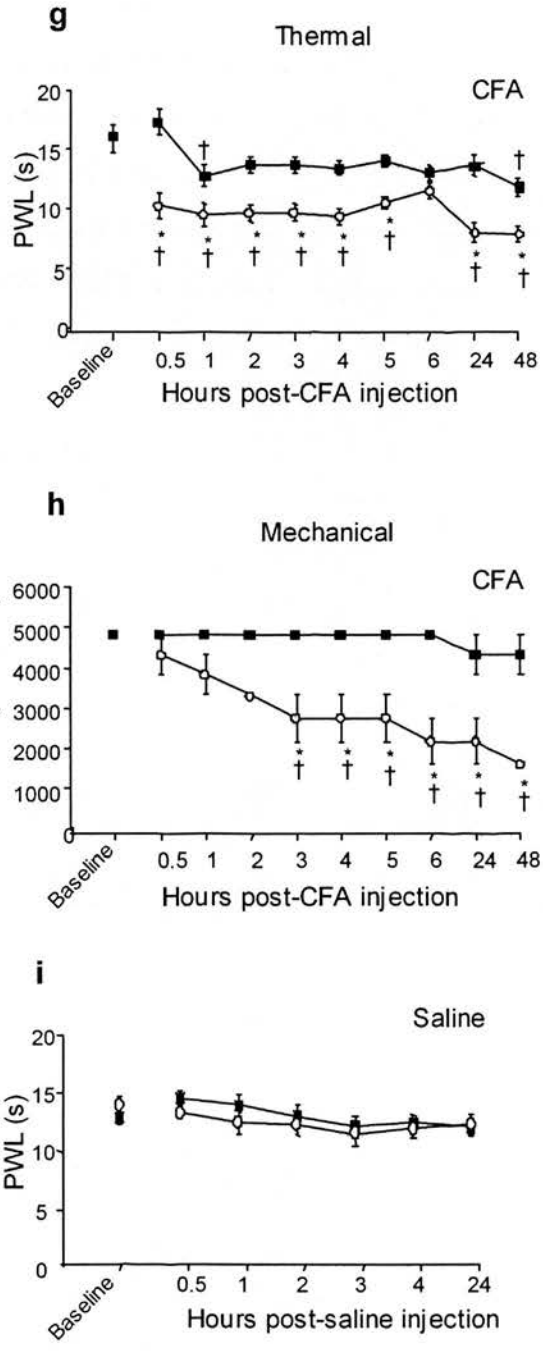

d
Thermal
Lysolecithin
PWL (s)
20
15
10
5
0
Baseline
4
5
7
12
13
15
Days post-surgery
e
Mechanical
Lysolecithin
PWT (mN/mm2)
6000
5000
4000
3000
2000
1000
0
Baseline
4
5
7
12
13
15
Days post-surgery
f
Cold
Lysolecithin
SPET (s)
20
15
10
5
0
Baseline
4
5
7
12
13
15
Days post-surgery

Figure 4.1 continued:

g, h, i) Timecourse of sensitisation following injection of CFA (g, h) or saline (i) into the hindpaw. Behavioural data in (g) and (i) show paw withdrawal latency (PWL; s) to a noxious thermal stimulus, (h) shows paw withdrawal threshold (PWT; mN/mm^2) to graded mechanical stimuli. Data show baseline values and measurements taken up to 48 hours following injection of CFA or saline to right hindpaw, mean ± SEM, n=6 animals. Ipsilateral paw (O) values show development of behavioural sensitisation following treatment with CFA but not saline, with limited effects on contralateral (■) paw values. Cold allodynia was not observed after CFA.

* indicates significant ($p<0.05$) ipsilateral-contralateral differences, as measured by Student's t-test for thermal data, and Wilcoxon test for mechanical data

† indicates significant difference ($p<0.05$) from pre-injection baseline values, as measured by One-Way RM ANOVA followed by Dunnett's post-hoc test for thermal data, and Friedman ANOVA on Ranks followed by Dunn's post-hoc test for mechanical data.


g
Thermal
CFA
PWL (s)
20
15
10
5
0
Baseline
0.5 1 2 3 4 5 6 24 48
Hours post-CFA injection
h
Mechanical
CFA
PWT (mN/mm²)
6000
5000
4000
3000
2000
1000
0
Baseline
0.5 1 2 3 4 5 6 24 48
Hours post-CFA injection
i
Saline
PWL (s)
20
15
10
5
0
Baseline
0.5 1 2 3 4 24
Hours post-saline injection

4.2 TRPM8 activation as a strategy to investigate the molecular mediation of cooling-induced analgesia

4.2.1 Rationale

The TRPM8 receptor is a good candidate for a molecular mediator of analgesia produced by innocuous cooling, as it is activated by innocuous cold temperatures, and is expressed in sensory neurons. (For a fuller discussion, see Introduction section 1.26). The role of this receptor in cooling-induced analgesia was investigated by examining the effect of TRPM8 activation on behavioural reflex sensitivity in awake, behaving animals. The CCI model of neuropathic pain was used as the principal model of a sensitised pain state, and behavioural reflex thresholds to noxious thermal stimuli (Hargreaves' test) and punctate mechanical stimuli (Von Frey filaments) were measured.

4.2.2 Chemical activation of TRPM8

Temperature stimuli may activate other transduction mechanisms in addition to TRPM8 and can produce additional physiological effects, such as cold-induced vasoconstriction. Therefore the effect of TRPM8 activation was initially investigated using chemical agonists. The two best-characterised TRPM8 agonists are menthol and icilin. These compounds are structurally unrelated: icilin is a 1,2,3,6-tetrahydropyrimidine-2-one (Wei, 1983), whereas menthol is a cyclic alcohol. Both structures are shown in Figure 4.2. Icilin was our first choice agonist, as it is the TRPM8 agonist with the highest potency and efficacy (Behrendt et al., 2004; McKemy et al., 2002). Of the various menthol stereoisomers, (-)-menthol has the highest potency (Bandell et al., 2006; Behrendt et al., 2004). Efficacy and potency data for icilin and menthol are shown in Table 4.1.

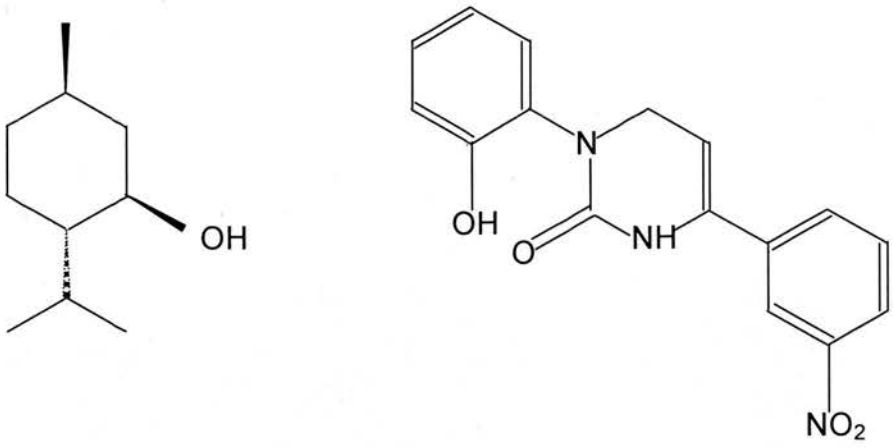


Figure 4.2: Structure of (-)-menthol (left-hand side) and icilin (right-hand side).

Drug	Efficacy as % Icilin Effect	EC_{50} (μM)
Icilin	100	0.2 ± 0.1
(-)-menthol	~71 ± 2	4.1 ± 1.3
(+)-menthol	~68 ± 4	14.4 ± 1.3

Table 4.1: Efficacy and potency data for TRPM8 agonists

Data adapted from Behrendt et al., 2004.

4.3 Effects of peripheral activation of TRPM8

4.3.1 Topical administration of TRPM8 agonists is analgesic in sensitised pain states

For the least invasive method of application and to model potential clinical usage, icilin and menthol were administered topically to the paws by placing awake rats in a waterbath containing 1 cm deep drug solution, to cover the feet, for a 5 minute period. Rats were unrestrained to minimise stress, and the waterbath was large enough to allow movement. Drug solution was maintained at 30 °C approximate skin temperature) to avoid any effects of local temperature changes. The ability of both icilin and menthol to exert effects when applied topically is known (Wei, 2003, Wei, 2005, Williams & Barry, 2004), so topical application is a sensible strategy.

After 5 minutes, icilin (80 μM) caused a striking reversal of the behavioural reflex sensitisation to thermal and mechanical stimuli that occurs ipsilateral to CCI. Icilin produced a significant increase from ipsilateral baseline values ($p<0.05$) and abolished ipsilateral-contralateral significant differences for up to 25 minutes following the application period. There was a mean percentage reversal of ipsilateral-contralateral differences of 82.4 ± 4.3% for thermal and 59.3 ± 5.2% for mechanical values (mean ± SEM, calculated over 10 – 25 minutes following drug administration). In contrast, there was no effect on the sensitivity of the uninjured contralateral paw (Figure 4.3a). Vehicle (0.2% DMF in water) had no effect on either paw, which demonstrates that the effect was not due to either vehicle or to the testing procedure (Figure 4.3b). To further investigate the effect of icilin, experiments were carried out using different concentrations of drug, under the same conditions. Concentration-dependent effects of icilin on ipsilateral thermal and mechanical analgesia were observed between 2.5 to 500 μM, as shown in Figure 4.3c.

I then investigated whether the same effects were produced by topical application of a second TRPM8 agonist, menthol. I initially used (-)-menthol, as this is suggested to have the highest potency at TRPM8 of the menthol isomers, and found that topical application of (-)-menthol (1R, 2S, 5R-(-)-menthol, 4 mM in water with 25% or 80% ethanol) produced a similar reversal of ipsilateral sensitisation in CCI animals (Figure 4.3d, shown for 80% ethanol vehicle), with no effect of either vehicle. As

with icilin, the analgesic effects appeared somewhat more marked on thermal than mechanical responses, although it is difficult to directly compare the results from these different tests with each other as they represent different types of data (parametric and non-parametric). The stereoisomers isomenthol (1S, 2R, 5R-menthol) and (+)-menthol (1S, 2R, 5S (+)-menthol) are slightly less potent agonists of TRPM8 – for EC_{50} data see Table 4.1 (Bautista et al., 2006; Behrendt et al., 2004). Initial tests of isomenthol at a concentration of 4 mM in 25% ethanol vehicle produced no significant effects on behavioural thresholds (data not shown), and therefore higher concentrations were tested. At concentrations of 8 mM and 16 mM, isomenthol and (+)-menthol also produced a reversal of sensitised responses, although to a lesser extent than (-)-menthol (Figure 4.3e). Solubilisation of these higher concentrations required a vehicle with a higher percentage of ethanol (80%) and therefore the 4mM (-)-menthol concentration was repeated with this new vehicle, and is shown as such in Figure 4.3d in order to compare with the data on (+)-menthol and isomenthol.

Neither icilin (Figure 4.3f) nor (-)-menthol (data not shown) had any effect in naïve animals, in agreement with the lack of effect on contralateral values. This suggests that the analgesic effect produced by these compounds is specific to the sensitised state.

4.3.2 Topical application of high doses of TRPM8 activators produces hyperalgesia

To further examine the concentration-response effect of icilin, the effects of topical application of much higher concentrations were examined, testing concentrations of up to 5 mM icilin. It was not possible to use the large 400 ml volumes used for waterbath application with such high concentrations, and therefore a different method was used. Rats were lightly anaesthetised, and the drug applied in small foot-tubes (modified 15 ml centrifuge tubes) which covered the paw without constricting it. As before, drug was maintained at approximately 30°C for 5 minutes. There was no effect of vehicle applied in this way, demonstrating that there was no lasting effect of the vehicle or the anaesthetic procedure. 5 mM icilin produced increased reflex sensitivity, both ipsilateral and contralateral to CCI and in naïve

animals (Table 4.2), that was statistically significant in mechanical and (after a delay) thermal tests. 2.5 mM icilin produced a trend towards sensitisation, although this was not significant, whereas 1 mM had no effect. This hyperalgesic effect was distinct from the analgesia produced by low concentrations of icilin, as it was not specific to a sensitised state and may be due to weak interaction of the drug with other targets or non-specific actions.

4.3.3 TRPM8 activators are only effective when applied to the area of injury

To investigate whether it is necessary that TRPM8 activators be applied directly to the area innervated by the injured nerve in order to have an effect, rather than to any area of the skin, I examined the effect of applying icilin only to the contralateral paw in CCI animals. Icilin (80 μM in water with 0.2% DMF) was topically applied in the small foot-tubes used for application of high doses, but was only applied to contralateral paw. There was no significant effect of drug on either ipsilateral or contralateral behavioural reflex sensitivity: mean ± SEM percentage reversals of ipsilateral-contralateral differences, calculated over 15-30 min following application, were -1.2 ± 8.6% for thermal and 0.0 ± 0.0% for mechanical values. In broad terms this result shows that in order for topical icilin to have an effect, it must be applied to the area from which sensitisation originates.

4.3.4 Activation of TRPM8 by cool temperatures produces mechanical analgesia

To investigate whether temperature activation of TRPM8 produced analgesia, I investigated the effects of paw immersion in waterbaths at temperatures from 10-22°C for 5 minutes. Only mechanical thresholds were measured, as the physical cooling of the paw would obviously interfere with the effectiveness of the heat stimulus used in the Hargreaves' test. The same procedure as waterbath administration of icilin was followed using water at different temperatures. Initial experiments which performed the first measurement 5 minutes after removal from the waterbath saw no change in mechanical threshold. However, further experiments which measured mechanical threshold immediately after removal from the waterbath, observed a statistically significant mechanical analgesia ipsilateral to CCI

after immersion in temperatures between 16 – 20°C, which was fully recovered by 5 minutes post-waterbath. This is shown in Figure 4.3g, using measurements taken immediately after removal from the waterbath. Therefore temperatures in the range of TRPM8 activation do produce a mechanical analgesia, supporting our findings with chemical agonists, and suggesting a TRPM8-mediated mechanism of analgesia. The reason for the much shorter effect of temperature is probably the recovery of local skin temperature, and resultant "switching off" of the TRPM8 receptor, whereas chemical agonists that penetrate the skin can exert a more prolonged activation of TRPM8.

Recordings from a subcutaneous thermistor probe in anaesthetised animals showed that deep skin temperatures were approximately 0.5 °C above bath temperatures after 5 minutes in similar conditions. This demonstrates that the bath temperature in these experiments does approximate to the subcutaneous temperature, although the stronger blood flow in unanaesthetised animals will probably result in higher deep skin temperatures in the unanaesthetised state.

In contrast to the effect of temperatures between 16 and 20°C, immersion temperatures below 14°C elicited withdrawal reflexes of the ipsilateral paw for the duration of the immersion period, in agreement with the temperature range for noxious cold sensation (Chery-Croze, 1983; Chen et al., 1996; Wolf & Hardy, 1941).

4.3.5 Topical icilin activates a subpopulation of slowly conducting afferents

Icilin is expected to activate TRPM8-expressing afferents. To investigate this, in vivo recordings of saphenous nerve afferents following topical application of icilin were carried out. The nerve was dissected to produce small-number preparations of afferents, with conduction velocities of up to 2.6 ms^{-1} - representing C- and Aδ-fibre afferents (Harper & Lawson, 1985). Small numbers of fast-conducting innocuous mechanosensitive afferents (presumed large A fibres) were present in each preparation; these were not analysed but were used to identify the receptive field area of the preparation by response to an innocuous brush stimulus. Drug or vehicle was applied to the receptive field via a soaked gauze patch.

Icilin (200 μM in water with 0.2% DMF) applied to the receptive field caused a significant increase in firing frequency in approximately 21.6% (40 out of 185) of

recorded fine afferents with a mean 7-fold increase in firing frequency from baseline of 4.5 ± 2.5 spikes per minute to 31.6 ± 3.4 spikes per minute ($p<0.05$, One-Way ANOVA on Ranks), and a mean time to peak effect of 3.3 ± 0.5 minutes. The mean time to peak effect of icilin is consistent with the onset of behavioural analgesia observed within 5 minutes of icilin application. Recovery was consistently observed, and similar results were obtained from both hairy and glabrous skin. A typical recording is shown in Figure 4.3i. There was no discernible effect of vehicle, as shown in Figure 4.3h indicating that the activation of afferents is specific to the drug. Icilin effects were apparent for up to 20 minutes following application of the icilin-soaked gauze patch. The effect of icilin on TRPM8 channels in vitro, where icilin activation is followed by extensive desensitisation (Andersson et al., 2004; Chuang et al., 2004), does therefore not appear to translate to a similar rapid desensitisation of afferent neuron firing in vivo. The majority of afferents activated by icilin showed conduction velocities <2.2 ms^{-1}, indicating that these were C fibres (Harper & Lawson, 1985). Only 5% showed higher conduction velocities, and, the highest conduction velocity recorded was 2.6 ms^{-1}, which is at the lower end of the Aδ fibre conduction velocity range, described by Harper & Lawson as being between 2.2 to 14 ms^{-1} (Harper & Lawson, 1985). In order to ascertain whether the effect of icilin was specific to slowly-conducting afferents, separate recordings were made of fast-conducting mechanoreceptors. These were identified by conduction velocities of 6.8-20 ms^{-1}, consistent with Aβ and Aδ fibres, and by response to innocuous mechanical brushing and stroking of the receptive field. These afferents were found to be unaffected by icilin, as shown in Figure 4.3j Therefore icilin appears to predominantly activate C fibre neurons. A summary of these results is provided in Table 4.3.

Figure 4.3 Peripheral TRPM8 activation and moderate cooling are analgesic following CCI

a, b) Behavioural data from CCI animals show that: (a) topical application of icilin (80 μM) significantly reversed ipsilateral sensitisation of paw withdrawal responses, with no effect on contralateral threshold, but (b) topical application of vehicle (0.2 % DMF in water) had no significant effect on behavioural reflexes.

Graphs show mean ± SEM (from an n of 6 animals) PWL (s) to noxious heat and PWT (mN/mm^2) to graded mechanical stimuli, before and following topical application (5 min paw immersion in a shallow 30 °C water bath) of icilin or vehicle to paws.

○: ipsilateral paw values, ■: contralateral paw values. * indicates significant ($p<0.05$) ipsilateral-contralateral differences, as measured by Student's t-test for thermal data, and Wilcoxon test for mechanical data; † indicates a significant ($p<0.05$) difference from pre-drug baseline values, as measured by One-Way RM ANOVA followed by Dunnett's post-hoc test for thermal data, and Friedman ANOVA on Ranks followed by Dunn's post-hoc test for mechanical data.

c) Concentration-response curve for percentage reversal (mean ± SEM) of ipsilateral sensitisation produced by topical icilin in CCI animals, for thermal (○) or mechanical (◆) tests, plotted on a logarithmic scale. Values calculated over 10-15 min following 5 min immersion in water bath containing concentrations of 2.5, 10, 50, 200 and 500 μM icilin, n of 6 animals for each concentration.

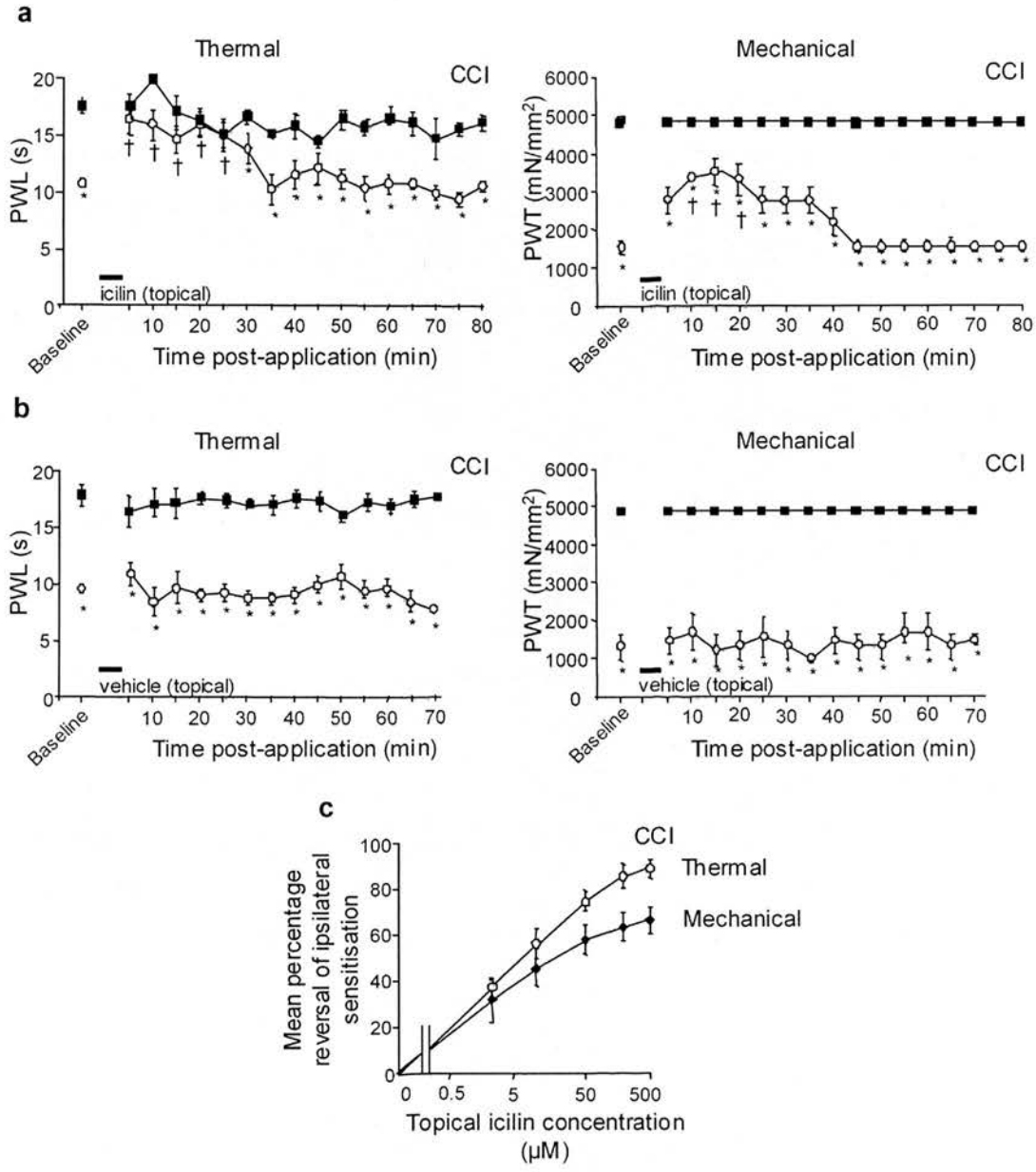

a
Thermal
CCI
PWL (s)
icilin (topical)
Baseline
Time post-application (min)
Mechanical
CCI
PWT (mN/mm²)
icilin (topical)
Baseline
Time post-application (min)
b
Thermal
CCI
PWL (s)
vehicle (topical)
Baseline
Time post-application (min)
Mechanical
CCI
PWT (mN/mm²)
vehicle (topical)
Baseline
Time post-application (min)
c
CCI
Thermal
Mechanical
Mean percentage reversal of ipsilateral sensitisation
Topical icilin concentration (μM)

Figure 4.3 continued:

d) Behavioural data from CCI animals show that topical (-)-menthol (4 mM in water with 80% ethanol) significantly reversed ipsilateral paw sensitisation, with no effect on contralateral threshold. The vehicle had no discernible effect on reflex responses (data not shown).

Graphs show mean ± SEM (n=6) PWL (s) to noxious heat and PWT (mN/mm^2) to graded mechanical stimuli, before and following topical application (5 min paw immersion in a shallow 30°C water bath) of (-)-menthol to paws.

○: ipsilateral paw values, ■: contralateral paw values. * indicates significant ($p<0.05$) ipsilateral-contralateral differences, as measured by Student's t-test for thermal data, and Wilcoxon test for mechanical data; † indicates a significant ($p<0.05$) difference from pre-drug baseline values, as measured by One-Way RM ANOVA followed by Dunnett's post-hoc test for thermal data, and Friedman ANOVA on Ranks followed by Dunn's post-hoc test for mechanical data.

e) Reversal of ipsilateral sensitisation by paw immersion in (-)-menthol at 4 mM, and by higher concentrations of the less potent stereoisomers (+)-menthol and isomenthol (8 and 16 mM). Values were calculated over 10-25 min following paw immersion from experiments. † indicates significant differences from pre-drug baseline ($p<0.05$).

f) Data from naive animals show that topical application of icilin (80 μM) has no discernible effect on behavioural reflex thresholds. Graphs show mean ± SEM (from an n of 6 animals) PWL (s) to noxious heat and PWT (mN/mm^2) to graded mechanical stimuli, before and following topical application (5 min paw immersion in a shallow 30°C water bath) of icilin to paws.

■: naive paw values. † indicates a significant ($p<0.05$) difference from pre-drug baseline values, as measured by One-Way RM ANOVA followed by Dunnett's post-hoc test for thermal data, and Friedman ANOVA on Ranks followed by Dunn's post-hoc test for mechanical data.

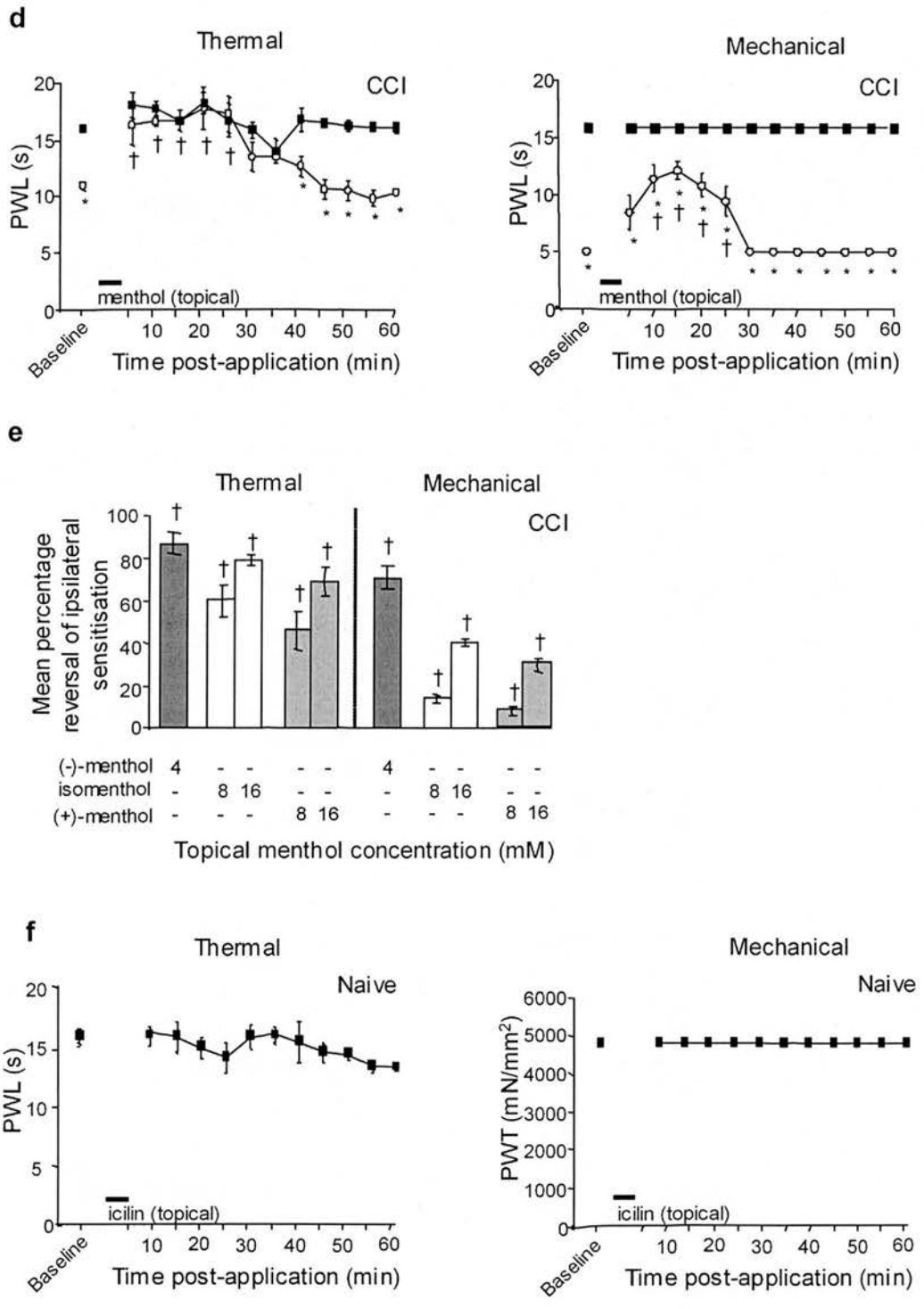

d
Thermal
CCI
PWL (s)
menthol (topical)
Baseline
Time post-application (min)
Mechanical
CCI
PWL (s)
menthol (topical)
Baseline
Time post-application (min)
e
Thermal
Mechanical
CCI
Mean percentage reversal of ipsilateral sensitisation
(-)-menthol
isomenthol
(+)-menthol
Topical menthol concentration (mM)
f
Thermal
Naive
PWL (s)
icilin (topical)
Baseline
Time post-application (min)
Mechanical
Naive
PWT (mN/mm²)
icilin (topical)
Baseline
Time post-application (min)

Figure 4.3 continued:

g) Behavioural data from CCI animals show the mechanical analgesic effect on ipsilateral paw of brief (5 min) paw immersion at indicated temperature range.
Graph shows mean ± SEM (n=6) PWT (mN/mm^2) to graded mechanical stimuli measured over 5 min following paw immersion at different temperatures. O: ipsilateral paw values, ■: contralateral paw values. * indicates significant ($p<0.05$) ipsilateral-contralateral differences, measured by Wilcoxon test; † indicates a significant ($p<0.05$) difference from pre-immersion baseline, measured by Friedman ANOVA on Ranks followed by Dunn's post-hoc test; § denotes spontaneous withdrawal responses.

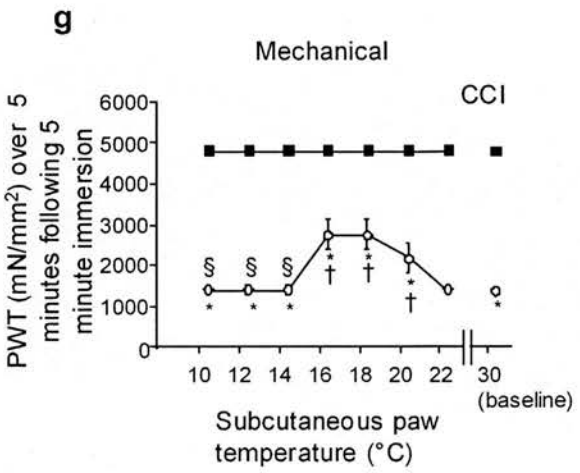
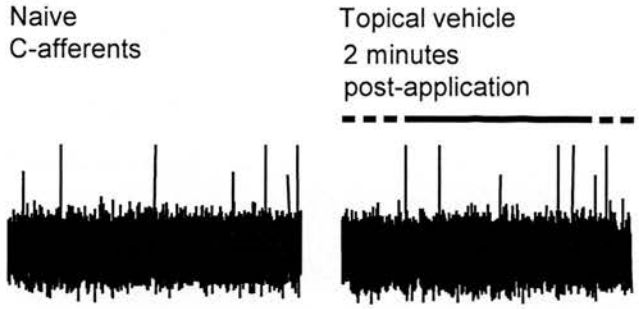

g
Mechanical
CCI
PWT (mN/mm2) over 5 minutes following 5 minute immersion
6000
5000
4000
3000
2000
1000
0
§
§
§
†
†
†
*
10 12 14 16 18 20 22 30
(baseline)
Subcutaneous paw temperature (°C)

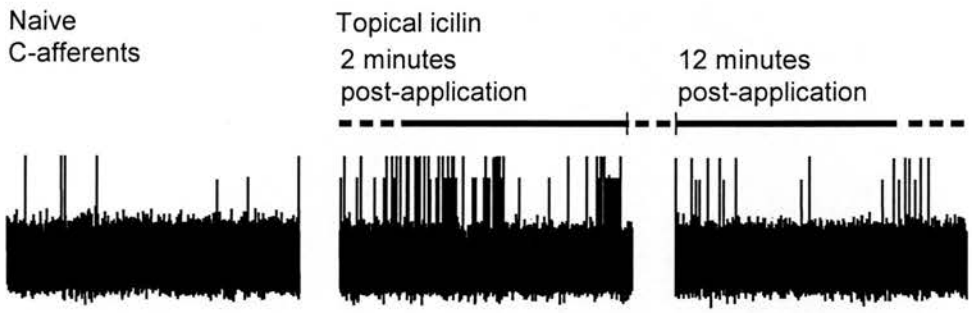
Figure 4.3 continued:

h, i, j) Electrophysiological recordings from small-number preparations of saphenous afferents. (h): shows a recording of C-fibre afferents before and following topical application of vehicle to the receptive field area. (i): shows an electrophysiological recording of icilin-responsive C-fibre afferents fibres before, 2 minutes (peak effect) and 12 minutes (recovery) following topical application of icilin (200 μM) to the receptive field area; observations typical of 40 similar recordings. (j): shows a recording of A-fibre afferents before and following topical application of icilin (200 μM) to the receptive field area, with no discernible effects on firing frequency.

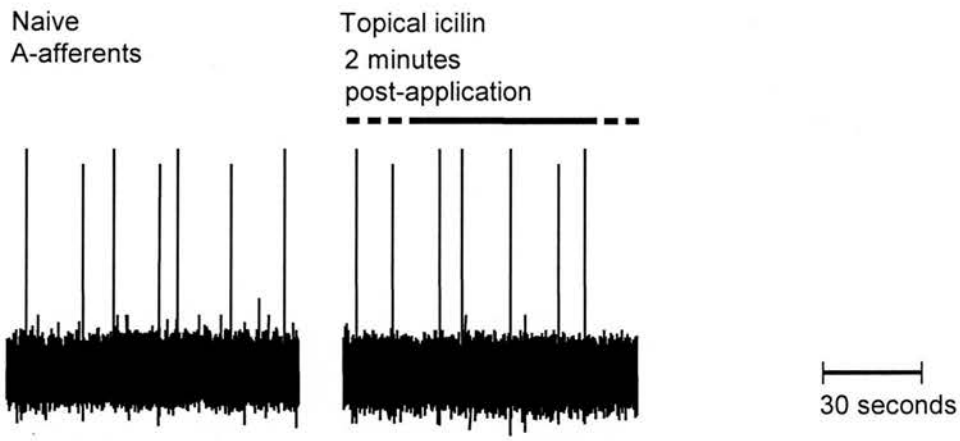
h


Naive
C-afferents
Topical vehicle
2 minutes
post-application

i


Naive
C-afferents
Topical icilin
2 minutes
post-application
12 minutes
post-application

j


Naive
A-afferents
Topical icilin
2 minutes
post-application
30 seconds

Reflex response sensitivity at different times after icilin application to paw						
Thermal PWL (s)						
Drug Conc-entration (μM)	**Naïve**		**CCI Ipsi**		**CCI Con**	
Pre-drug Baseline	15.5 ± 0.2		9.7 ± 0.2		16.4 ± 0.2	
	15 mins	**50 mins**	**15 mins**	**50 mins**	**15 mins**	**50 mins**
0 (vehicle)	15.1 ± 1.2	14.9 ± 0.8	9.8 ± 1.1	9.7 ± 0.9	15.2 ± 0.6	15.1 ± 0.7
1000	15.1 ± 1.2	14.8 ± 0.9	16.4 ± 0.8†	8.6 ± 0.2	16.1 ± 0.5	15.2 ± 0.4
2500	14.9 ± 0.3	13.2 ± 0.8	16.4 ± 0.9†	8.4 ± 0.6	16.8 ± 0.9	15.3 ± 0.7
5000	15.3 ± 0.5	13.1 ± 0.7§	16.7 ± 1.0†	7.2 ± 0.6§	16.1 ± 0.9	13.4 ± 0.7§
Mechanical PWT (mN/mm^2)						
	Naïve		**CCI Ipsi**		**CCI Con**	
Pre-drug Baseline	4830.6 ± 0.0		805.7 ± 26.3		4793.6 ± 37.5	
	15 mins	**50 mins**	**15 mins**	**50 mins**	**15 mins**	**50 mins**
0 (vehicle)	4830.6 ± 0.0	4830.6 ± 0.0	805.7 ± 26.3	805.7 ± 26.3	4830.6 ± 0.0	4830.6 ± 0.0
1000	4830.6 ± 0.0	4830.6 ± 0.0	702.8 ± 185.3	702.8 ± 185.3	4530.1 ± 300.5	4530.1 ± 300.5
2500	4830.6 ± 0.0	4830.6 ± 0.0	651.9 ± 193.0	651.9 ± 193.0	4454.9 ± 245.9	4454.9 ± 245.9
5000	3828.6 ± 316.8§	4830.6 ± 0.0	412.2 ± 24.3§	736.0 ± 26.3	2040.2 ± 115.2§	4830.6 ± 0.0

Table 4.2: Analgesic effects of topically administered icilin against neuropathic sensitisation revert at very high concentrations to a general nociceptive effect

Significant effects of drug on reflex responses are indicated: † denotes significant increase from baseline indicating analgesic effect of icilin, § denotes significant decrease from baseline values indicating hyperalgesic effects, One-Way RM ANOVA followed by a Dunnett's test for thermal, Friedman ANOVA on Ranks followed by a Dunn's test for mechanical values.

Afferent Type	Baseline Firing Frequency (spikes per minute)	Activity after Icilin at Peak effect (spikes per minute)	Activity at (Partial) Recovery (spikes per minute)	Activity after Vehicle Application (spikes per minute)	Time till Peak Effect (min)
Icilin-responsive slowly-conducting afferents (1.3–2.6ms^{-1}), n=40, 21.6 %	4.5 ± 2.6	31.6 ± 3.4*	9.5 ± 1.9*	7.7 ± 3.3	3.3 ± 0.5
Icilin-unresponsive slowly-conducting afferents (1.3–2.6ms^{-1}), n=145	7.5 ± 0.8	10.3 ± 1.4	10.0 ± 2.1	6.6 ± 1.0	-
Fast-conducting afferents (6.8 – 20ms^{-1}), n=43	9.2 ± 2.4	13.5 ± 2.6	-	-	-

Table 4.3: Summary of afferent recordings following topical icilin application.
* denotes significant difference from baseline in firing frequency ($p<0.05$, ANOVA on Ranks).

4.4 Central administration of TRPM8 activators is analgesic in CCI and in other chronic pain models

4.4.1 Intrathecal administration of TRPM8 activators is analgesic in CCI animals

As TRPM8 is present on the central terminals of primary sensory neurons (Baccei et al., 2003; Tsuzuki et al., 2004; see also Figure 4.5f,) I investigated whether intrathecal application of TRPM8 activators would also produce analgesia.

200 μM icilin (in saline with 0.2% DMF) produced robust reversal of CCI-induced behavioural reflex sensitisation in thermal and mechanical tests. There was a significant increase from baseline, and reversal of ipsilateral-contralateral differences, for up to 55 min (Figure 4.4a). An equivalent volume of vehicle (0.9% saline with 0.2% DMF) had no effect (data not shown). A second TRPM8 agonist, (-)-menthol, produced similar effects . An initial dose of 25 nmol (-)-menthol (in saline) produced no discernible effect on thermal or mechanical threshold. However, a higher dose of 200 nmol (-)-menthol (in saline vehicle), caused a significant reversal of ipsilateral sensitisation in CCI rats, lasting up to 40 min (Figure 4.4b). The higher doses of alternative menthol stereoisomers tested topically could not be tested intrathecally, due to the higher ethanol concentrations of vehicle required to dissolve these. In conclusion, both menthol and icilin produced significant analgesia when applied topically and intrathecally. However, because of the higher potency and efficacy of icilin (Behrendt et al., 2004; McKemy et al., 2002), and the possibility of additional effects of menthol (Eccles, 1994; Macpherson et al., 2006), most further experiments used icilin to activate TRPM8.

I then tested for a dose-response relationship, in similar experiments to those performed topically, using doses of 10, 2.5, 0.5 and 0.125 nmol. Icilin produced dose-dependent analgesic effects on ipsilateral reflex sensitivity in both thermal and mechanical tests that were statistically significant at 0.125 nmol and increased to almost complete reversal of sensitisation by 10 nmol. Figure 4.4c shows the dose-response curve, plotted on a logarithmic scale. From this graph, the calculated maximal effects of icilin were similar for thermal and mechanical thresholds (91.6 ±

9.9 and 82.6 ± 6.8% reversal of sensitisation, respectively), as were ED_{50} values (dose for 50% of maximal effect: 0.17 ± 0.02 and 0.31 ± 0.02 nmol, respectively).

4.4.2 Intrathecal icilin is also effective in other chronic pain models

In further experiments it was investigated whether the sensitised behaviours caused by other pain models were susceptible to icilin-induced analgesia.

Investigation of the CFA model of chronic inflammatory pain required work-up of this model, to investigate the timecourse of peak sensitivity (as described in section 4.1). The effect of intrathecal icilin (10 nmol in saline) in this model was then measured, and icilin again significantly reversed behavioural reflex sensitisation (Figure 4.4d), demonstrating that icilin is an effective analgesic in inflammatory as well as neuropathic pain states.

Following on from this, the lysolecithin model of demyelination-induced pain was investigated, which has some similarities to neuropathic pain models, but also important differences (Wallace et al., 2003). Because this model represents another, distinct example of chronic pain, and because demyelination-induced pain states are associated with abnormal processing of sensory cold (Ochoa & Yarnitsky, 1994), this was an interesting model in which to further investigate the effects of icilin. This model was set up and a timecourse established as described in section 4.1, and then the effect of intrathecal icilin was tested at the period of peak sensitivity. There were no obvious acute effects of lysolecithin, or any behavioural effects beyond those reported. Lysolecithin is another name for lysophosphatidylcholine, which has been identified as a TRPM8 agonist (Vanden Abeele et al., 2006). Presumably however, the administration of a high dose of lysolecithin to the outside sheath of the sciatic nerve primarily causes demyelination of the axons travelling within the nerve, rather than causing effects on the peripheral TRPM8 receptors. Intrathecal icilin (10 nmol in saline) significantly reversed reflex sensitisation in this model (Figure 4.4e). This demonstrates the effectiveness of icilin as an analgesic against a variety of sensitised pain states, both inflammatory and neuropathic, adding to its potential clinical utility, and also demonstrates that there is no apparent desensitisation of icilin's analgesic effect caused by the application of lysolecithin to the nerve. Table 4.4 shows the mean percentage reversals of ipsilateral sensitisation produced by 10 nmol icilin in

the CCI, CFA and lysolecithin models, calculated over 15-30 minutes following injection. The effect of icilin is strongest in the CCI model and weakest in the CFA model, but the differences are not substantial (perhaps with the exception of the relatively poor reversal of CFA-induced mechanical sensitisation).

In further experiments on lysolecithin-demyelinated animals, the effect of icilin on the SPET test of cold allodynia was measured. Intrathecal icilin (10 nmol) significantly reduced withdrawal responses to 4 °C cold as measured by the SPET test (Figure 4.4f). This shows that cold allodynia can also be reversed by TRPM8 activation, thereby distinguishing the function of subpopulations of cold-sensitive afferents.

As with topical application, intrathecal icilin had no discernible effect in normal animals - with no injury model performed - (Figure 4.4g), again indicating that the analgesic effect of icilin is specific to sensitised pain states.

Pain Model	Mean % reversal of thermal sensitisation (± SEM)	Mean % reversal of mechanical sensitisation (± SEM)
CCI	84.7 ± 6.0	76.8 ± 5.3
CFA	66.0 ± 7.5	42.6 ± 2.8
Lysolecithin	69.2 ± 5.1	63.3 ± 6.4

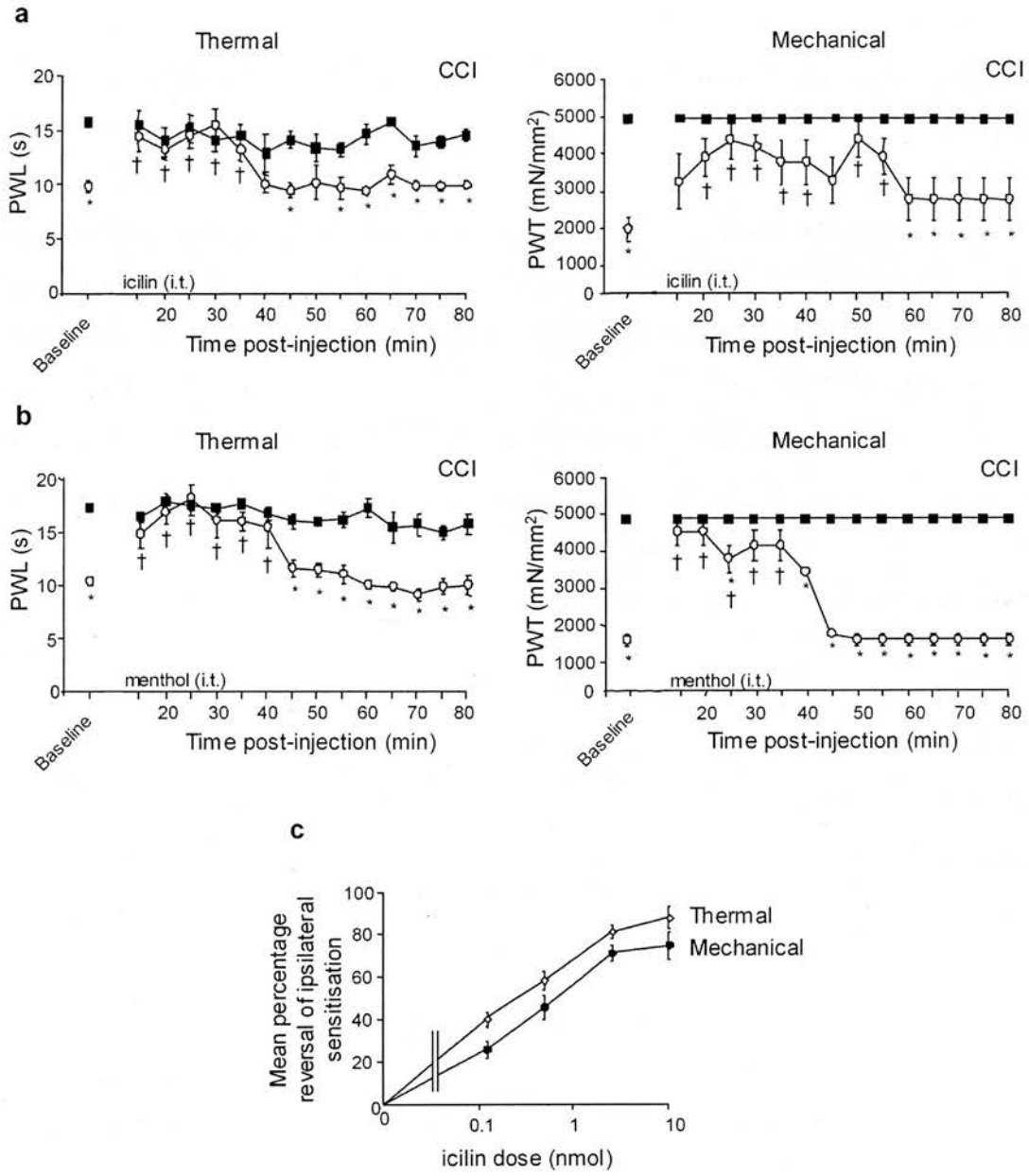
Table 4.4: Reversal of sensitisation by intrathecal icilin (10 nmol) in different pain models.

Figure 4.4 Central TRPM8 activation is also analgesic following CCI

a, b) Behavioural data from CCI animals show that intrathecal injection of both (a) icilin (10 nmol) and (b) (-)-menthol (200 nmol) significantly reversed thermal and mechanical reflex sensitisation ipsilateral to CCI, with no effect on contralateral threshold.

Data show mean ± SEM (from an n of 6 animals) PWL (s) to a noxious thermal stimulus and PWT (mN/mm^2) to graded mechanical stimuli. ○: ipsilateral paw values, ■: contralateral paw values. * indicates significant ($p<0.05$) ipsilateral-contralateral differences, as measured by Student's t-test for thermal data, and Wilcoxon test for mechanical data; † indicates a significant ($p<0.05$) difference from pre-drug baseline values, as measured by One-Way RM ANOVA followed by Dunnett's post-hoc test for thermal data, and Friedman ANOVA on Ranks followed by Dunn's post-hoc test for mechanical data.

c) Dose-response curve shows the mean percentage reversal ± SEM of ipsilateral sensitisation produced by intrathecal icilin in CCI animals, for thermal (○) or mechanical (◆) tests, plotted on a logarithmic scale. Values calculated over 20-35 min following injection with doses of 0.125, 0.5, 2.5 and 10 nmol icilin, with an n of 6 animals for each dose.


a
Thermal
CCI
PWL (s)
icilin (i.t.)
Baseline
Time post-injection (min)
Mechanical
CCI
PWT (mN/mm²)
icilin (i.t.)
Baseline
Time post-injection (min)
b
Thermal
CCI
PWL (s)
menthol (i.t.)
Baseline
Time post-injection (min)
Mechanical
CCI
PWT (mN/mm²)
menthol (i.t.)
Baseline
Time post-injection (min)

c
Mean percentage reversal of ipsilateral sensitisation
Thermal
Mechanical
icilin dose (nmol)

Figure 4.4 continued:

d, e, f) Behavioural data show that intrathecal injection of icilin (10 nmol) in (d) CFA-injected animals, and (e) lysolecithin-treated animals significantly reversed the thermal and mechanical reflex sensitisation of the ipsilateral hindpaw in both of these pain models, and the cold allodynia which develops ipsilateral to lysolecithin treatment.

Data show mean ± SEM (from an n of 6 animals) PWL (s) to a noxious thermal stimulus, PWT (mN/mm^2) to graded mechanical stimuli, and suspended paw elevation time (SPET (s) to 4°C cold water over a 20 s immersion. ○: ipsilateral paw values, ■: contralateral paw values. * indicates significant ($p<0.05$) ipsilateral-contralateral differences, as measured by Student's t-test for thermal data, and Wilcoxon test for mechanical data; † indicates a significant ($p<0.05$) difference from pre-drug baseline values, as measured by One-Way RM ANOVA followed by Dunnett's post-hoc test for thermal data, and Friedman ANOVA on Ranks followed by Dunn's post-hoc test for mechanical/cold data.

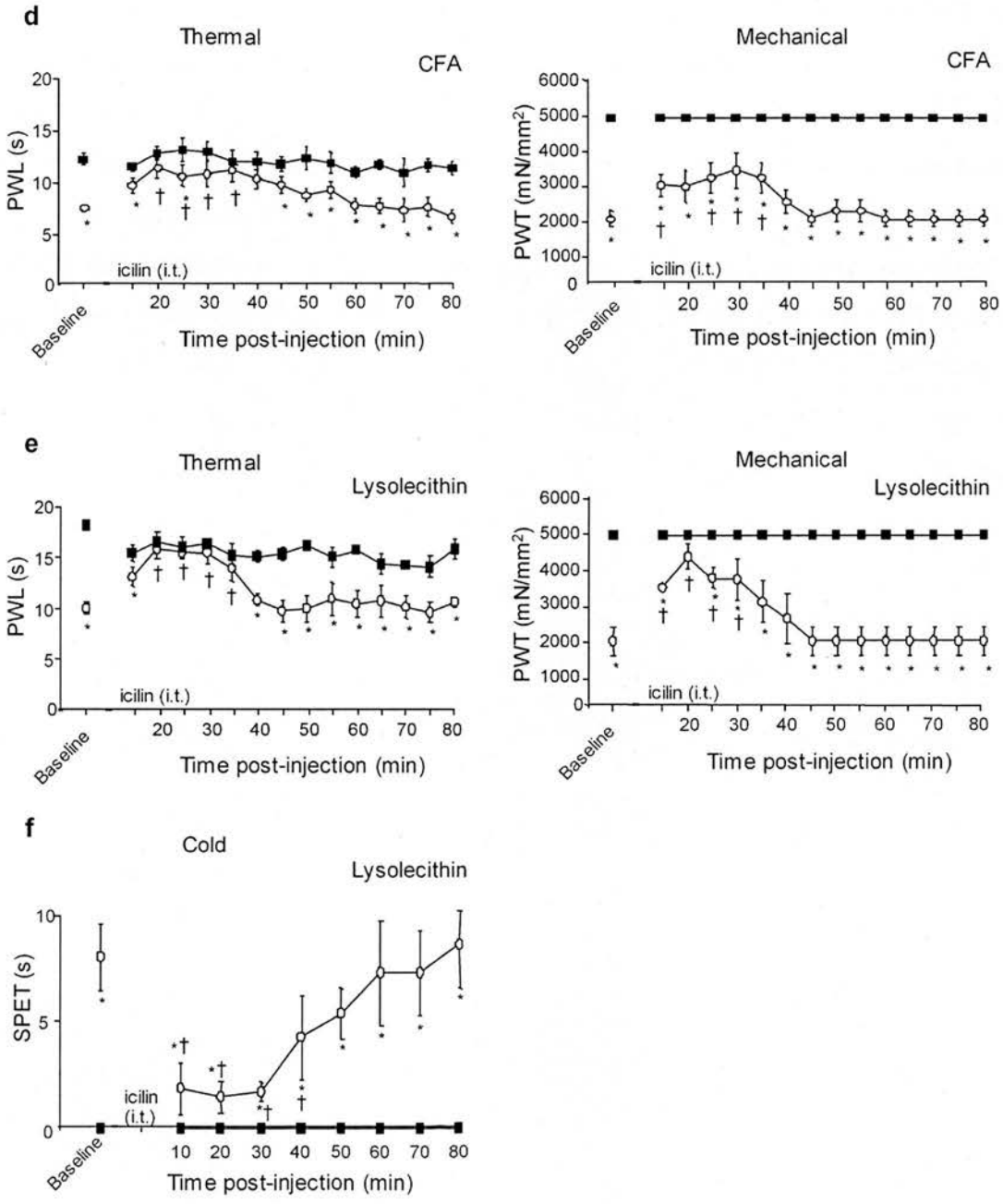
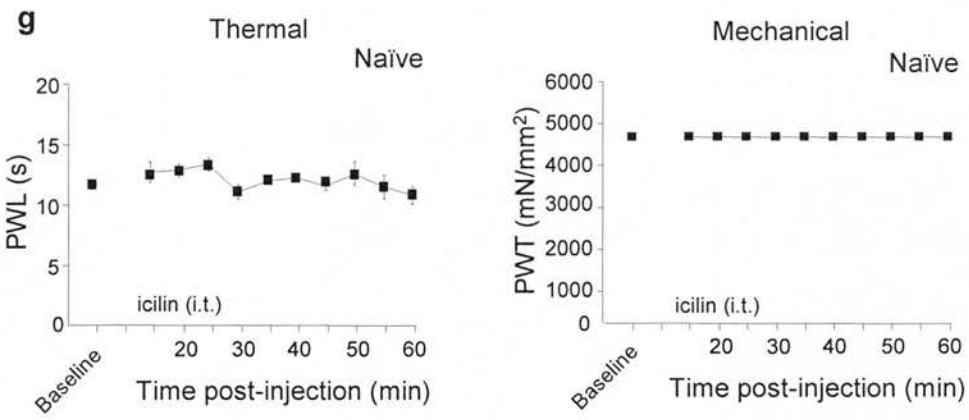

d
Thermal
CFA
PWL (s)
icilin (i.t.)
Baseline
Time post-injection (min)
Mechanical
CFA
PWT (mN/mm²)
icilin (i.t.)
Baseline
Time post-injection (min)
e
Thermal
Lysolecithin
PWL (s)
icilin (i.t.)
Baseline
Time post-injection (min)
Mechanical
Lysolecithin
PWT (mN/mm²)
icilin (i.t.)
Baseline
Time post-injection (min)
f
Cold
Lysolecithin
SPET (s)
icilin (i.t.)
Baseline
Time post-injection (min)

Figure 4.4 continued:

g) Behavioural data show that intrathecal injection of icilin (10 nmol) had no effect in naïve animals.

Data show mean ± SEM (from an n of 6 animals) PWL (s) to a noxious thermal stimulus and PWT (mN/mm^2) to graded mechanical stimuli. ■: naive paw values.


g
Thermal
Naïve
PWL (s)
20
15
10
5
0
icilin (i.t.)
Baseline
20
30
40
50
60
Time post-injection (min)
Mechanical
Naïve
PWT (mN/mm²)
6000
5000
4000
3000
2000
1000
0
icilin (i.t.)
Baseline
20
30
40
50
60
Time post-injection (min)

4.5 Localisation of TRPM8 in afferents and superficial dorsal horn

4.5.1 Western blot demonstration of TRPM8 presence in DRG and spinal cord

The presence and localisation of TRPM8 in DRG and spinal cord were investigated by immunoblotting. Tissue was homogenised in Laemmli lysis buffer and boiled to break non-covalent bonds in protein structures and generate soluble, linearised proteins, and the resulting homogenate was separated by SDS-PAGE. Immunoblots were then probed with a rabbit polyclonal antibody raised to TRPM8 residues 278-292 and 1090-1104 (human). Initial experiments separated proteins on a 4 – 12% gradient gel, and resulting immunoblots showed bands at 128 kDa - the predicted molecular weight of TRPM8 - and further, somewhat weaker bands at approximately 170, 60 and 50 kDa. These additional bands could represent breakdown fragments of the TRPM8 protein (or in the case of the 170 kDa band, breakdown of TRPM8 multimers) or non-specific interactions of the antibody. In order to improve the immunoblot results, a more rapid homogenisation process was employed (collecting tissue directly onto ice-cold foil and weighing and homogenising immediately), in order to decrease protease breakdown of TRPM8 to fragments. In one trial 6 M urea and 2 M thiourea was included in the lysis buffer to help break non-covalent bonds; however, there was no effect of such treatment on the TRPM8 banding pattern, and therefore in all further blots tissue was homogenised in normal Laemmli buffer. Samples were heated to 70°C rather than 100°C, as higher temperatures can cause aggregation of proteins which contain significant stretches of hydrophobic amino acids, such as membrane proteins like TRPM8 (Chemicon online guide to Western blotting). Proteins were separated on 3 – 8% gradient gels, which are designed to give a better separation of higher molecular weight proteins. Under these conditions, I observed a single, strong band at 128 kDa, with very much weaker bands at 170, 60 and 50 kDa (Figure 4.5a).

To further assess any importance of these additional bands and to assess antibody specificity, antigen-pre-absorption and antisense-knockdown controls were carried out. Both controls were consistent with this antibody specifically recognising TRPM8 protein at approximately 128 kDa under the conditions used. Pre-incubation of the antibody with membranes from COS 7 cells transfected with TRPM8

expression plasmid abolished the band at 128 kDa, whereas sham pre-absorption with membranes from cells transfected with empty vector did not (Figure 4.5a). Antisense knockdown was carried out by intrathecal delivery of TRPM8 antisense oligonucleotide from implanted osmotic minipumps in naïve (non-CCI) rats over 5 days to downregulate expression of the TRPM8 receptor protein. Intrathecally delivered fluorescent-labelled oligonucleotides have been shown to effectively penetrate the DRG, as soon as 4 hours after initial delivery (Lai et al., 2002); hence intrathecal delivery is a reasonable method of delivering oligonucleotides to the DRG. Antisense pre-treatment also resulted in almost complete knockdown of the 128 kDa band (Figure 4.5a), whereas mis-sense control oligonucleotide was ineffective (see below). The faint bands, at 50 and 60 kDa at least, remained present in each case and so are likely to represent non-specific interactions of the antibody under these conditions. The housekeeping enzyme GAPDH (36 kDa), used as a loading control, was evenly present in each lane. As a further control, it was shown that the related sensory TRP receptor, TRPV1, was unaffected by treatment with the TRPM8 antisense reagent (Figure 4.5a).

In further support of these results, an alternative antibody, primarily used for immunohistochemistry, produced a similar band at 128 kDa, with almost complete knockdown in antisense-treated tissue (Figure 4.5d). The similarity in the patterns for these two antibodies, and the abolition of the band at 128 kDa by both antibody pre-absorption and antisense knockdown of TRPM8, very strongly suggests that the band at 128 kDa on Western blots does indeed represent the TRPM8 protein. For this reason, only this band is shown on further blots, rather than whole gels.

4.5.2 TRPM8 increases ipsilateral to CCI

Following nerve injury, there was a marked increase in expression of the 128 kDa TRPM8-immunoreactive band specifically in ipsilateral, but not contralateral DRG (Figure 4.5b), whereas immunoreactivity for GAPDH was unaltered. As assessed by semi-quantitative densitometry and expressed as a percentage (± SEM) of the GAPDH signal, the TRPM8 signal was 80.7 ± 4.1% in ipsilateral DRG, which is significantly different to both contralateral (49.3 ± 3.2%) and naive (50.7 ± 2.7%) ($p<0.05$, One-Way ANOVA, n=5-6) signals.

L4-5 spinal cord lysates showed that TRPM8 immunoreactivity was present centrally. There was no alteration in TRPM8 immunoreactivity in these lysates (staining density as a percentage (± SEM) of the GAPDH signal was 31.2 ± 5.8% in ipsilateral spinal cord, 26.4 ± 6.8% in contralateral cord and 36.2 ± 3.1% in naïve cord, n=6). The lack of differences could be due to low levels of TRPM8 present in the spinal cord, which are simply too low to show a significant difference between ipsilateral and contralateral levels. Therefore I prepared a crude particulate fraction (by centrifugation at 11 000g for 45 min) to enrich the membrane component. In such preparations, consistent increases in expression ipsilateral to injury were observed (Figure 4.5b). There are no appropriate loading controls for membrane or particulate fractions and therefore TRPM8 densitometry values are expressed as a percentage of naïve values. Ipsilateral staining density was 198.0 ± 6.7% of naïve, contralateral was 125.0 ± 7.1, which represents a significant difference between ipsilateral and contralateral, and between ipsilateral and naïve staining ($p<0.05$, One-Way ANOVA, n=5).

In contrast, in DRG lysates from CFA-injected and control saline injected animals, there was no difference between ipsilateral and contralateral DRG, or between CFA-injected and saline-treated animals (Figure 4.5c). Staining density as a percentage of GAPDH was 46.3 ± 4.9% ipsilateral to CFA, 50.4 ± 5.2% contralateral to CFA, 43.2 ± 5.6% ipsilateral to saline, and 42.7 ± 6.8% contralateral to saline treatment.

4.5.3 Immunohistochemical analysis of TRPM8 expression

Immunohistochemistry was carried out using a rabbit polyclonal antibody raised to TRPM8 residues 656-680 (rat) (Brauchi et al., 2004). Antibody concentration and protocol was optimised and the specificity of the antibody was assessed by antigen pre-absorption and antisense knockdown controls. The labelling observed in a discrete subpopulation of DRG cells was abolished following pre-incubation of the antibody with the peptide antigen (Figure 4.5e). When processed with the antigen-pre-incubated antibody no positive cells were seen, compared with 5.3 ± 0.4 TRPM8-positive cells per 500 μm^2 DRG section following sham treatment of antibody, and 5.1 ± 0.5 TRPM8-positive cells per 500 μm^2 DRG section with the

untreated antibody, mean ± SEM, counted over 12 sections in each condition. When run on immunoblots, the antibody also labelled a single band at approximately 128 kDa in naïve DRG tissue that was abolished either by pre-absorption with the peptide antigen or by prior 5 day intrathecal infusion of TRPM8 antisense reagent (Figure 4.5d). GAPDH immunoreactivity was unaffected by either treatment. This reflects the result obtained with antisense-treated tissue and the alternative antibody used above (Figure 4.5a).

4.5.4 TRPM8 localisation on afferent terminals in spinal cord

Immunohistochemistry showed TRPM8 protein in the superficial dorsal horn of the spinal cord, like the afferent C fibre marker, peripherin (Amaya et al., 2000), as shown in Figure 4.5f,g. To examine the origin of this spinal TRPM8 immunoreactivity, a dorsal root rhizotomy was performed at spinal cord levels L2-L6 and tissue taken 8 days later for immunohistochemical analysis. Dorsal rhizotomy removes peripheral afferent input to the spinal cord; proteins remaining in the spinal cord following this procedure are, generally speaking, due to expression by neurons intrinsic to the spinal cord. The vast majority of TRPM8 immunoreactivity was lost ipsilateral to rhizotomy, indicating that spinal TRPM8 originates largely from afferents. Figure 4.5f shows a spinal cord section following rhizotomy, stained for TRPM8 and peripherin. Localisation of TRPM8 protein in the superficial dorsal horn is apparent in contralateral side, and this staining is virtually abolished ipsilateral to rhizotomy, together with peripherin expression. Fluorescence intensity was reduced by approximately 80-90% compared with control contralateral side, demonstrating a substantial decrease (although fluorescence intensity is non-linear and therefore cannot easily be correlated with protein expression).

4.5.5 TRPM8 increases in dorsal horn ipsilateral to CCI

Immunohistochemical staining of L4-L6 spinal cord sections from CCI animals confirmed that TRPM8 levels were increased in the dorsal horn ipsilateral to injury compared with contralateral control (an approximate increase in fluorescence

intensity of 70-80%), but retained a similar distribution in superficial laminae. Figure 4.5g shows a typical section immunostained for TRPM8 and neuronal nuclei.

4.5.6 TRPM8 expression alters in primary afferent subpopulations

To establish whether the increases in afferent TRPM8 expression occurred in specific subpopulations of DRG cells, I investigated TRPM8 co-localisation with neurofilament-200 (NF-200), which marks myelinated afferents (Lawson et al., 1984; Lawson & Waddell, 1991) and peripherin, which labels unmyelinated afferents (Amaya et al., 2000). In naïve rats, TRPM8 immunoreactivity was largely confined to a subpopulation of unmyelinated DRG cells (8.3 ± 0.2% of peripherin-positive cells; 34 of 408 cells) and only minimally expressed in myelinated, NF-200-positive cells (1.3 ± 0.5%; 6 of 445 cells). TRPM8-expressing peripherin-positive cells were small diameter (mean diameter 18.4 ± 0.7 μm). However, following CCI, TRPM8 expression was significantly increased ipsilaterally in both NF-200- and peripherin-positive cells to 7.9 ± 1.2% (31 of 390 cells) and 15.5 ± 0.8% (64 of 412 cells), respectively. Corresponding contralateral values were unaltered from naives at 2.0 ± 0.4 % (14 of 346 cells) and 9.2 ± 0.4% (42 of 452 cells) (Figure 4.5h,i,j,k). Data are taken from 3 CCI and 3 naïve animals, counted across 15 – 21 sections. The additional TRPM8-expressing NF-200-positive cells were small (mean diameter: 19.7 ± 0.8 μm), presumed Aδ myelinated neurons (Harper & Lawson, 1985). There were no significant differences in the diameters of NF-200- or peripherin-positive cells or in the numbers of NF-200- or peripherin-positive DRG cells per section, between ipsilateral, contralateral and naïve conditions.

Figure 4.5 TRPM8 immunoreactivity is present in DRG and spinal cord, arises from afferents and is increased ipsilateral to CCI

a) Immunoblots of DRG show whole gels with TRPM8 protein running at 128 kDa and additional faint bands at approximately 170, 60 and 50 kDa, in normal rat DRG with specific knockdown of the 128 kDa band in DRG from antisense-treated animals. Pre-incubation of TRPM8 antibody with membranes from TRPM8-expressing cells also removed the 128 kDa immunoreactive band, whereas sham-treatment had no effect. GAPDH loading controls are also shown. TRPV1 expression (single band at ~90 kDa) was unaltered in DRG from TRPM8 antisense-treated animals.

b) Immunoblots for TRPM8 protein show a clear increase in expression of the specific 128 kDa band in DRG ipsilateral (ipsi) to nerve injury compared to contralateral (con) and naive DRG. Levels of the housekeeping protein, GAPDH were unchanged. Spinal cord (SC) whole lysates showed no discernible changes in TRPM8 levels, however, increased levels were seen in crude particulate fractions ipsilateral to nerve injury.

c) Immunoblots for TRPM8 protein show no alteration in expression of the 128 kDa band in DRG ipsilateral or contralateral to CFA treatment, or between CFA and saline-injected animals. GAPDH is shown as a loading control.

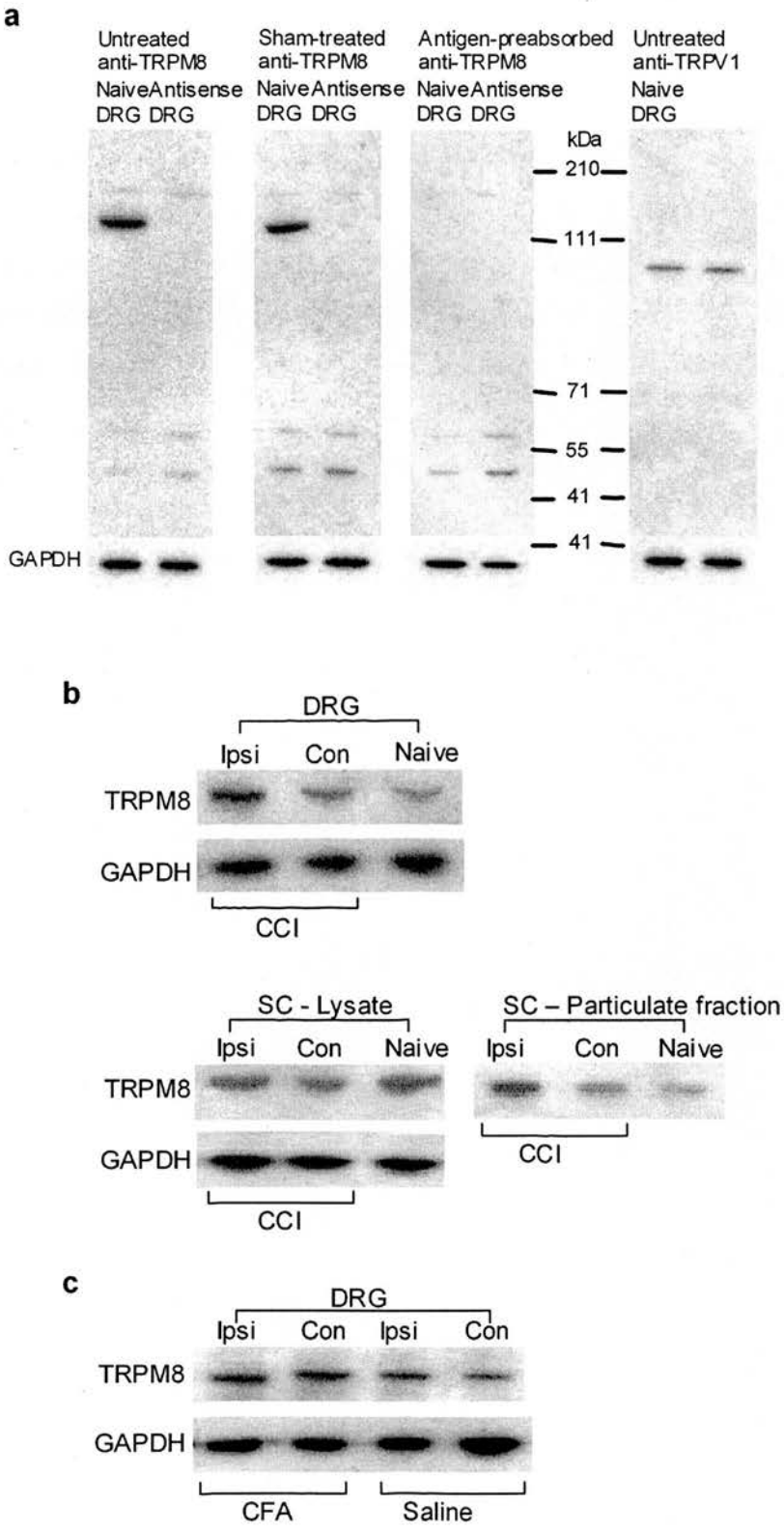
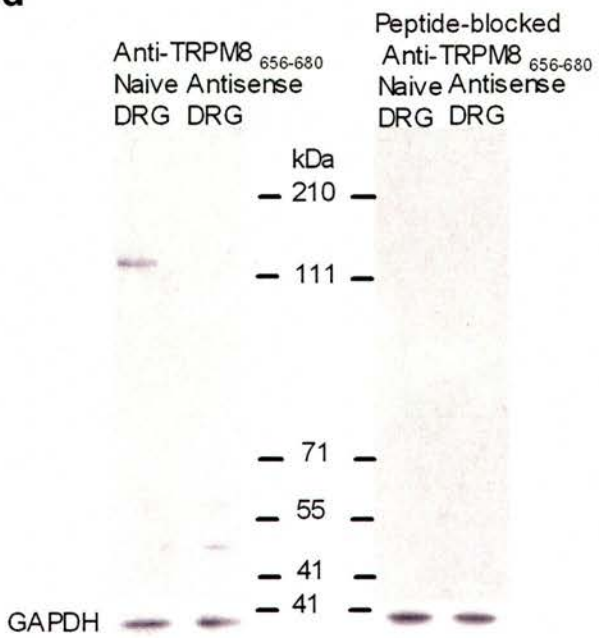

a
Untreated anti-TRPM8
Naive DRG
Antisense DRG
Sham-treated anti-TRPM8
Naive DRG
Antisense DRG
Antigen-preabsorbed anti-TRPM8
Naive DRG
Antisense DRG
Untreated anti-TRPV1
Naive DRG
kDa
210
111
71
55
41
41
GAPDH
b
DRG
Ipsi
Con
Naive
TRPM8
GAPDH
CCI
SC - Lysate
Ipsi
Con
Naive
TRPM8
GAPDH
CCI
SC – Particulate fraction
Ipsi
Con
Naive
CCI
c
DRG
Ipsi
Con
Ipsi
Con
TRPM8
GAPDH
CFA
Saline

Figure 4.5 continued:

d) Immunoblots of DRG from naïve or TRPM8 antisense-treated rats probed with the TRPM8 antibody used for immunohistochemistry (raised to residues 656-680) also show a specific band at ~128 kDa, which was removed by pre-incubation of the antibody with the antigenic peptide or by TRPM8 antisense treatment.

e) DRG sections stained with anti-TRPM8 antibody (red) following pre-incubation with peptide antigen ("blocked", right-hand side) or sham treatment ("sham", left-hand side). Staining is completely abolished by pre-incubation with peptide antigen. To-pro stained nuclei are shown in blue.

d


Anti-TRPM8 656-680
Naive DRG
Antisense DRG
Peptide-blocked Anti-TRPM8 656-680
Naive DRG
Antisense DRG
kDa
210
111
71
55
41
41
GAPDH

e

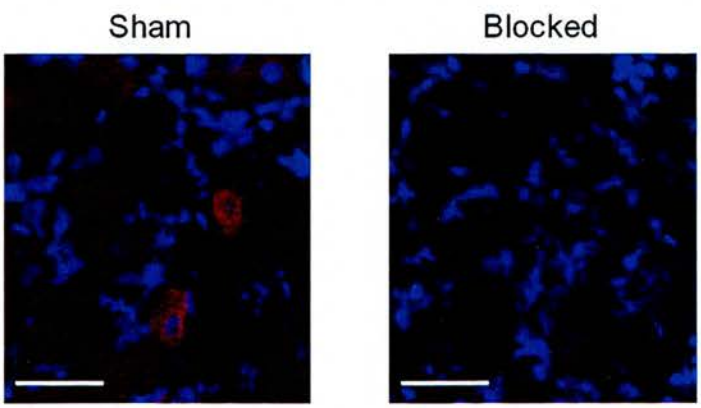
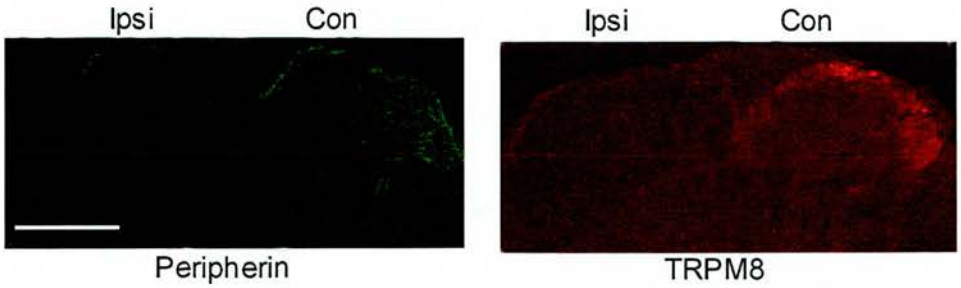

Sham
Blocked

Figure 4.5 continued:

f) L5 spinal cord dorsal horn section taken 8 days after dorsal rhizotomy at levels L2-L6, immunostained for peripherin (green) and TRPM8 (red) visualised under a confocal microscope. Both peripherin and TRPM8 staining are strongly reduced ipsilateral (ipsi) to rhizotomy compared with contralateral (con). Scale bar is 500 μm.
g) L5 spinal cord dorsal horn section from CCI animal, immunostained for TRPM8 (red) and neuronal nuclei (green), visualised under a confocal microscope. TRPM8 increases ipsilateral (ipsi) to CCI with no change in distribution. NeuN levels are unchanged. Scale bar is 500 μm.

f

Rhizotomy



g

CCI

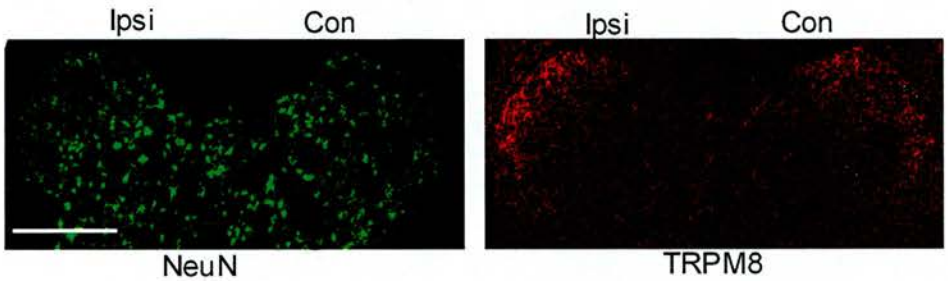
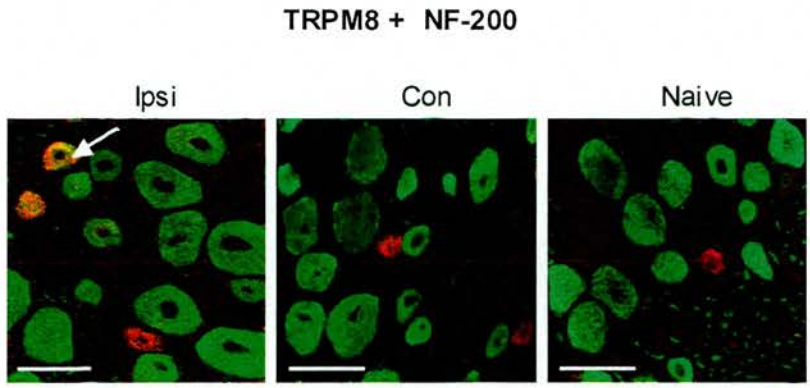


Figure 4.5 continued:

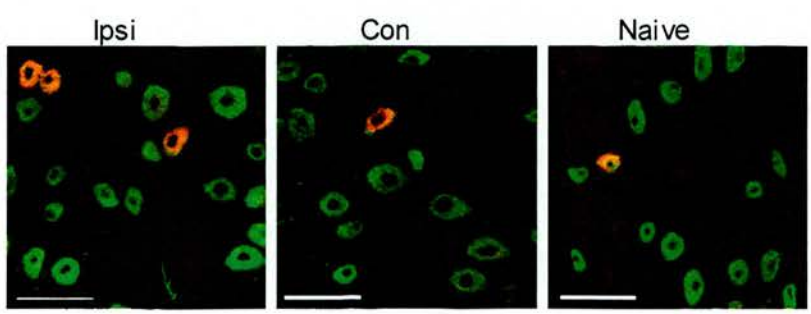
h, i) show DRG sections ipsilateral (ipsi) or contralateral (con) to CCI and from naive animals, immunostained for TRPM8 (red) with (h): the myelinated A-fibre marker NF-200 (green) or (i): the C-fibre marker peripherin (green), visualised under a confocal microscope. In naïve animals, TRPM8 is mainly located in peripherin-positive C-fibres with little or no apparent expression in myelinated (NF-200) cells. However, ipsilateral to CCI, TRPM8 expression increases markedly in small NF-200-positive cells (mean diameter 19.7 ± 0.8μm). A similar, but less marked increase in TRPM8:peripherin co-expression was also observed. Scale bar is 50μm.

j, k) show the percentage co-expression (mean ± SEM) for TRPM8:NF-200 and TRPM8:peripherin respectively; actual cell counts are shown above columns. Statistically significant increases in the percentage co-expression values are indicated (*, $p<0.05$, One-Way ANOVA).

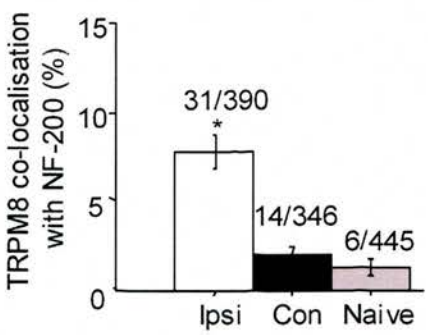
h


TRPM8 + NF-200
Ipsi
Con
Naive

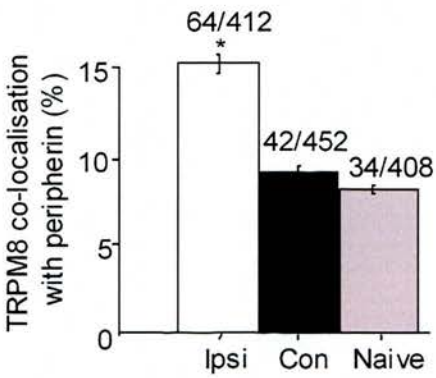
i


TRPM8 + peripherin
Ipsi
Con
Naive

j


TRPM8 co-localisation with NF-200 (%)
15
10
5
0
31/390
*
14/346
6/445
Ipsi
Con
Naive

k


TRPM8 co-localisation with peripherin (%)
15
10
5
0
64/412
*
42/452
34/408
Ipsi
Con
Naive

4.6 Molecular identification of TRPM8 as the mediator of icilin-induced analgesia

4.6.1 Abolition of icilin-mediated analgesia following TRPM8 knockdown

To verify the specific involvement of TRPM8 in icilin-induced analgesia I used an antisense knockdown strategy. TRPM8 antisense or mismatched control mis-sense oligonucleotides were delivered intrathecally by implanted osmotic minipumps over 13 days. As mentioned earlier (section 4.5.1), there is evidence that intrathecally delivered oligonucleotides can penetrate the DRG (Lai et al., 2002), which would be expected to affect peripheral levels of TRPM8. CCI surgery was performed at the same time as minipump implantation. The development of CCI-induced behavioural sensitisation was unaffected by either antisense or mis-sense treatment, with no difference in timecourse from standard CCI animals. This included development of withdrawal responses to 4°C cold. 9 - 11 days after CCI surgery, control CCI animals had a mean SPET value of 8.1 ± 0.5 s on ipsilateral paw, whereas corresponding values in antisense-treated CCI animals were 7.6 ± 0.6 s. This suggests that TRPM8 is not required for the development of cold allodynia, in agreement with results from other groups (Katsura et al., 2006).

However, the reversal of behavioural sensitisation normally produced by topical application of 80 μM icilin (as in Figure 4.3a) was largely abolished by treatment with antisense (Figure 4.6a), but not mis-sense (Figure 4.6b) oligonucleotides. Mean ± SEM percentage reversals of ipsilateral sensitisation over 10-25 minutes after icilin treatment in antisense- and mis-sense-treated animals were 7.7 ± 7.4% and 82.8 ± 6.9% respectively for PWL, and 9.4 ± 8.2% and 58.7 ± 8.2% for PWT; with mis-sense-treated, but not antisense-treated animals, retaining significant effects of icilin ($p<0.05$). Icilin tests were carried out between 9-13 days post-surgery, at peak CCI sensitisation and before depletion of minipumps. When minipumps were depleted (5 days after calculated end-time of pump), but animals were still neuropathic, a significant analgesic effect of icilin was restored in antisense animals (Figure 4.6c), to 83.7 ± 10.1% reversal of sensitisation for PWL, and 54.0 ± 7.2% for PWT.

4.6.2 Assessment of the efficacy of knockdown

Western blotting and peripheral afferent recordings were performed in order to assess the effectiveness and specificity of antisense knockdown. TRPM8 expression in both ipsilateral and contralateral DRG was greatly reduced by the antisense reagent and the increase in TRPM8 expression normally seen ipsilateral to nerve injury was prevented (Figure 4.6d). The mis-sense reagent had no effect (Figure 4.6d) showing TRPM8 expression similar to untreated animals (Figure 4.5a). In mis-sense treated animals, TRPM8:GAPDH percentage ratios were 77.9 ± 2.0% ipsilateral to CCI and 52.9 ± 2.1% contralateral, whereas in antisense-treated animals, values were much lower (19.8 ± 2.2% and 14.9 ± 2.1%, respectively, mean ± SEM, n=5), representing a significant difference from mis-sense values ($p<0.05$, One-Way ANOVA). As demonstrated in previous immunoblots, there was no effect of antisense treatment on levels of the related sensory TRP receptor, TRPV1 (Figure 4.5a).

To confirm that antisense knockdown of TRPM8 resulted in associated functional changes in afferents, saphenous nerve recordings were made from naïve animals receiving intrathecal delivery of TRPM8 antisense or mis-sense oligonucleotides, 4-5 days after insertion of the pump. These were performed in the same way and with the same icilin concentration as those described above in section 4.3.6. There was very little icilin-evoked increase in firing frequency in animals receiving antisense. Only 8.8% (3 out of 34) of recorded fibres showed a partial activation in response to the drug, increasing firing approximately 2-fold from a baseline of 5.8 ± 1.4 to 12.7 ± 0.6 spikes per minute, compared with the 7-fold increase observed in over 20% of fine afferents in naïve animals. In contrast, in mis-sense animals, icilin activated 25% (10 out of 40) of fine afferents, producing a 7-fold increase in firing frequency (from a baseline of 3.3 ± 0.7 to 23.1 ± 3.2 spikes per minute), similar to results from naïve animals. Typical examples of few-fibre recordings from mis-sense and antisense treated animals are shown in Figure 4.6e.

Similarly, in mis-sense-treated animals, topical (-)-menthol (4 mM in water with 25% ethanol) produced an approximately 8-fold increase in mean firing frequency (from 4.5 ± 2.9 to 38.9 ± 7.6 spikes per minute), activating 20% of fibres (35 identified afferents recorded). This compared with no obviously activated afferents in

antisense-treated animals (mean firing frequency 4.0 ± 1.8 spikes per minute at background, 4.8 ± 1.9 spikes per minute post-drug application, 28 identified afferents recorded). Example traces are shown in Figure 4.6f.

Attempts were made to measure responses to cool temperatures (between 12 and 18°C) of afferents in antisense and mis-sense- treated animals. However, there was great difficulty in maintaining the temperature of the receptive field of the rat hindpaw while recording from afferents in the nearby exposed saphenous nerve. Therefore experiments concentrated solely on responses to chemical agonists, which were easier to apply and which were used for the majority of behavioural studies.

To further investigate the specificity of TRPM8 antisense treatment, we recorded the responses of afferents to topically applied resiniferatoxin (1 mM in ethanol), which is a potent agonist of the heat-and-capsaicin receptor, TRPV1, and which is expected to activate a subpopulation of heat-sensitive polymodal nociceptive afferents, in a similar manner to capsaicin (Kenins, 1982). Resiniferatoxin was used in preference to capsaicin, as similar experiments had previously been conducted in the laboratory using resiniferatoxin. In support of the specificity of antisense treatment, resiniferatoxin-evoked responses were present in both antisense and mis-sense treated animals. In antisense-treated animals, resiniferatoxin evoked a 6-fold increase in firing frequency in activated afferents (from 4.5 ± 0.7 baseline to 25.9 ± 1.8 spikes per minute at peak response, 16 afferents activated out of 28 recorded), which was similar to responses in mis-sense-treated animals (showing a mean 5-fold change in firing frequency from 4.6 ± 2.8 to 24.8 ± 3.1 spikes per minute). Example traces are shown in Figure 4.6g.

In addition, the mechanoreceptive response to light tactile stimulation of the receptive field was observed to still be present in antisense-treated animals, supporting the specificity of antisense treatment.

Figure 4.6: Specific TRPM8 knockdown by antisense oligonucleotide prevents icilin-induced analgesia following CCI

a, b, c) Behavioural data show response to topical icilin in CCI animals treated with antisense (n=12) or mis-sense (n=10) oligonucleotides to TRPM8. (a): following treatment with antisense, icilin had no analgesic effect on ipsilateral behavioural reflex responses indicated by the persistence of ipsilateral-contralateral differences. (b): in contrast, following mis-sense treatment, icilin produced a significant reversal of sensitisation similar to untreated CCI animals. (c): 5 days after calculated end of antisense delivery, the analgesic effect of icilin was restored.

Data show mean ± SEM (from an n of 6 animals) PWL (s) to a noxious thermal stimulus and PWT (mN/mm^2) to graded mechanical stimuli.

○: ipsilateral paw values, ■: contralateral paw values. * indicates significant ($p<0.05$) ipsilateral-contralateral differences, as measured by Student's t-test for thermal data, and Wilcoxon test for mechanical data; † indicates a significant ($p<0.05$) difference from pre-drug baseline values, as measured by One-Way RM ANOVA followed by Dunnett's post-hoc test for thermal data, and Friedman ANOVA on Ranks followed by Dunn's post-hoc test for mechanical data.

d) Immunoblots of DRG tissue probed for TRPM8 and GAPDH protein levels following antisense knockdown of TRPM8, compared with mis-sense controls. The 128 kDa band for TRPM8 and, notably, the nerve-injury-induced ipsilateral increase in expression, was selectively reduced by antisense, but not mis-sense infusion; GAPDH levels were unchanged.

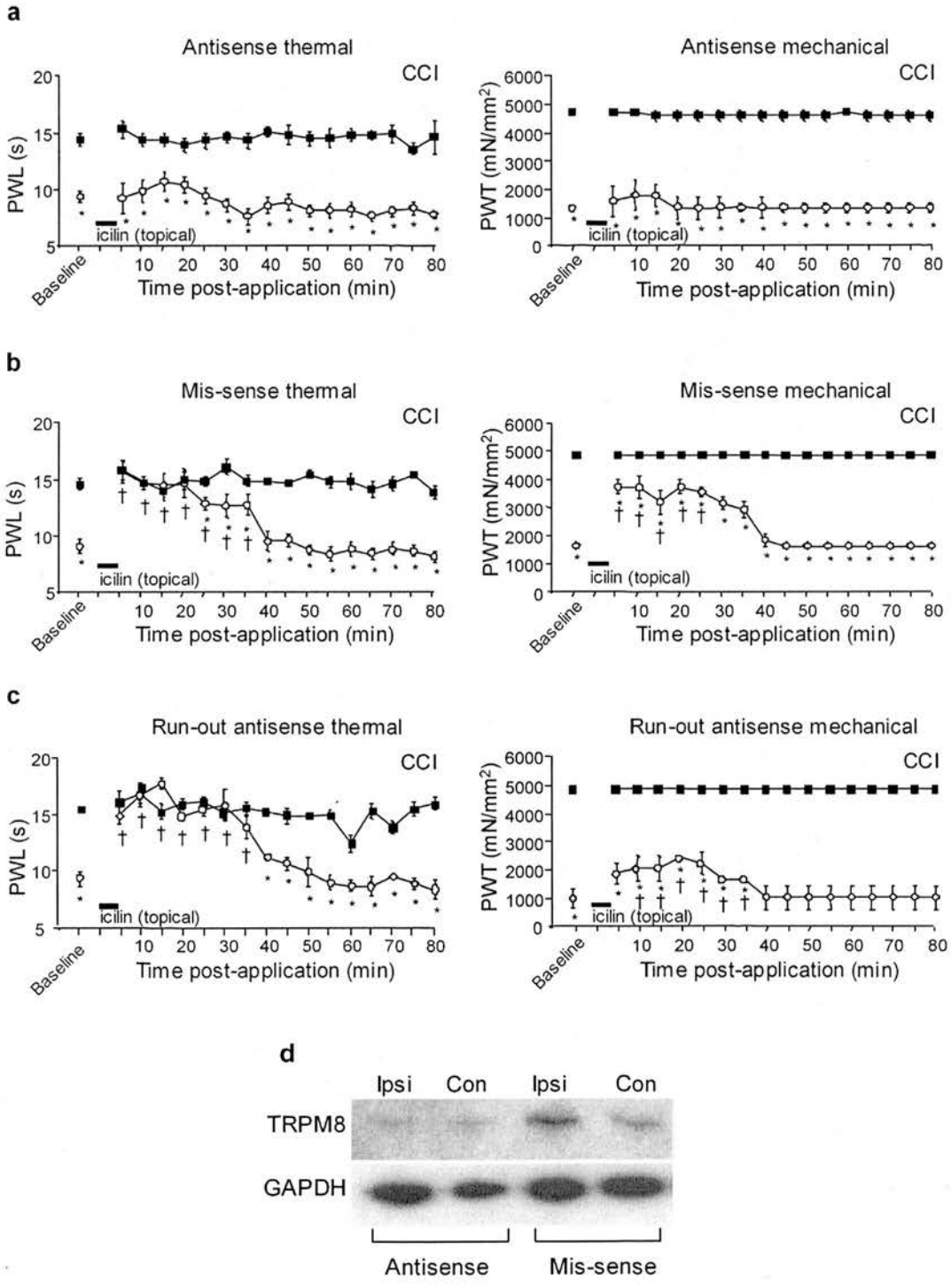

a
Antisense thermal
CCI
PWL (s)
icilin (topical)
Baseline
Time post-application (min)
Antisense mechanical
CCI
PWT (mN/mm2)
icilin (topical)
Baseline
Time post-application (min)
b
Mis-sense thermal
CCI
PWL (s)
icilin (topical)
Baseline
Time post-application (min)
Mis-sense mechanical
CCI
PWT (mN/mm2)
icilin (topical)
Baseline
Time post-application (min)
c
Run-out antisense thermal
CCI
PWL (s)
icilin (topical)
Baseline
Time post-application (min)
Run-out antisense mechanical
CCI
PWT (mN/mm2)
icilin (topical)
Baseline
Time post-application (min)
d
Ipsi
Con
Ipsi
Con
TRPM8
GAPDH
Antisense
Mis-sense

Figure 4.6 continued:

e, f) Electrophysiological recording from few-fibre preparations of C afferents in animals (without nerve injury) treated with mis-sense oligonucleotides or antisense oligonucleotides, a minimum of 4 days previously.
(e): topical icilin (200 μM) to the receptive field evoked an increase in firing in mis-sense animals similar to that evoked in naïve animals (Figure 4.3h), but had almost no effect in antisense treated animals. (f): similarly, topical (-)-menthol (4 mM) evoked an increase in firing in mis-sense treated, but not antisense treated animals.

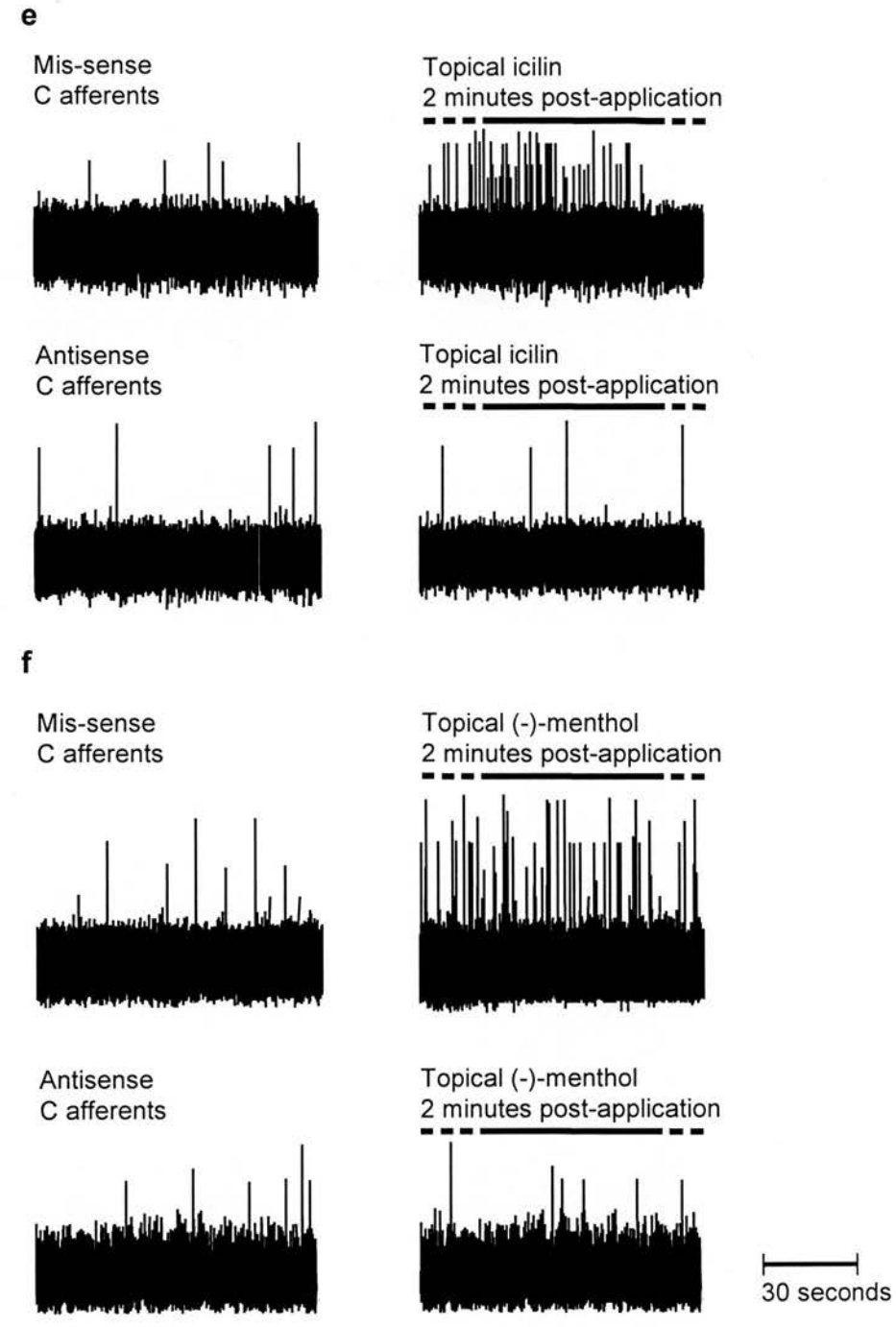
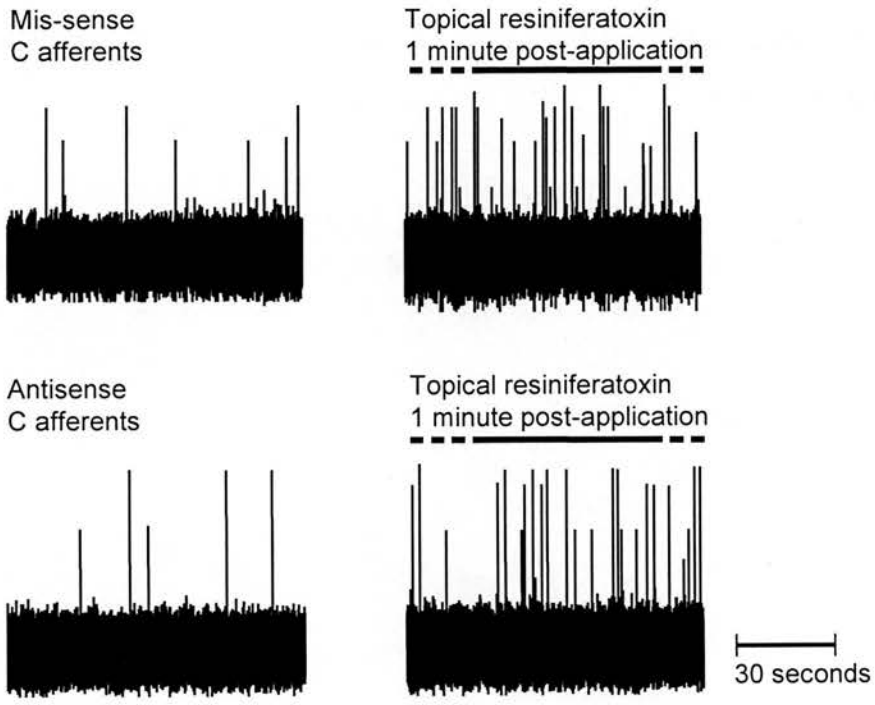

e
Mis-sense
C afferents
Topical icilin
2 minutes post-application
Antisense
C afferents
Topical icilin
2 minutes post-application
f
Mis-sense
C afferents
Topical (-)-menthol
2 minutes post-application
Antisense
C afferents
Topical (-)-menthol
2 minutes post-application
30 seconds

Figure 4.6 continued:

g) Electrophysiological recording from few-fibre preparations of C afferents in animals (without nerve injury) treated with mis-sense oligonucleotides or antisense oligonucleotides, a minimum of 4 days previously.
Topical application of the TRPV1 agonist resiniferatoxin (1 mM) to the receptive field evoked an increase in firing in both antisense and mis-sense treated animals, demonstrating that antisense treatment was specific to TRPM8.

g


Mis-sense
C afferents
Topical resiniferatoxin
1 minute post-application
Antisense
C afferents
Topical resiniferatoxin
1 minute post-application
30 seconds

4.7 Activation of TRPA1 is hyperalgesic and this effect is reversed by TRPM8 activation

4.7.1 Intrathecal and topical activation of TRPA1 produces hyperalgesia

The TRPA1 receptor has also been identified as cold-sensitive (Story et al., 2003), and icilin is also an agonist of TRPA1, albeit to a lesser extent than at TRPM8. To identify the effect of TRPA1 activation in vivo, and to establish whether or not there is a role for this receptor in icilin-induced analgesia, specific agonists of the TRPA1 receptor were administered in nerve-injured and naïve animals. In contrast to the effect of TRPM8 activators, TRPA1 activation produced a hyperalgesic effect, in both nerve-injured and naïve animals, in agreement with other groups (Bandell et al., 2004; Namer et al., 2005).

Intrathecal cinnamaldehyde (75 nmol in saline) produced sensitisation of thermal and mechanical behavioural reflexes in naïve animals. The same dose produced contralateral thermal and mechanical sensitisation in CCI animals, and produced an increase in ipsilateral thermal sensitisation, and a small, but significant increase in mechanical sensitisation (Figure 4.7a,b). A lesser effect was seen with a lower dose (25 nmol) in naïve animals: this evoked a mean percentage reduction ± SEM in baseline values of 17.8 ± 4.2 for thermal and 30.1 ± 3.7 for mechanical, compared with 35 ± 1.1% for thermal and 43 ± 3.8% for mechanical for the 75 nmol dose, over 20-30 minutes post-injection. The higher dose (75 nmol) was used in all further experiments as it produced a stronger effect. There was no effect of vehicle (0.9% saline). Co-injection of the broad spectrum TRP channel antagonist, ruthenium red (0.25 nmol in saline), which blocks TRPA1, but not TRPM8 (Macpherson et al., 2005; Weil et al., 2005) blocked the hyperalgesic effect produced by cinnamaldehyde, but not the analgesic effect of icilin, ipsilateral to CCI (Figure 4.7c). This data supports the idea that the hyperalgesic effect of cinnamaldehyde is mediated through TRPA1, whereas the analgesic effect of icilin is mediated through TRPM8. The further TRPA1 activating compounds, allicin and diallyl disulphide, which are pungent compounds derived from garlic (Bautista et al., 2005; Macpherson et al., 2005), also produced sensitisation in naïve animals. Intrathecal allicin (25 nmol) produced a mean percentage reduction from baseline values (over 15 – 25

minutes post-injection) of 33.2 ± 5.9%for PWL and 20.7 ± 8.2% for PWT. Similarly, diallyl disulphide (50 nmol) produced reductions (over 15 – 25 minutes post-injection) of 25.9 ± 7.0% for PWL and 28.5 ± 6.2% for PWT (shown in Figure 4.7d).

Cinnamaldehyde was also applied topically in small foot-tubes. The same concentration (1.5 mM in water) used for the higher dose of intrathecal cinnamaldehyde was utilised. This topical application also produced bilateral sensitisation of behavioural reflexes in naive animals, with a mean percentage reduction from baseline values (over 15 – 25 minutes) of 32.0 ± 8.6%in PWL and 20.2 ± 8.2% in PWT, (shown in Figure 4.7e).

4.7.2 TRPM8 activation reverses TRPA1-mediated sensitisation

To investigate the interaction between TRPM8 and TRPA1 activation, icilin (10 nmol) was intrathecally injected with cinnamaldehyde (0.75 nmol). Icilin blocked the sensitising effect of cinnamaldehyde in naïve animals (Figure 4.7f) so that overall, there was no observable change in sensory threshold. Further, icilin blocked contralateral sensitisation and produced a slight ipsilateral analgesia when co-injected with cinnamaldehyde in CCI animals (Figure 4.7g). The finding that TRPA1 agonists exert the opposite effect to icilin supports the findings of the antisense studies that icilin acts through TRPM8 to produce analgesia. The inhibitory effect of icilin on cinnamaldehyde-induced analgesia could be due to icilin activating TRPM8-expressing afferents and causing an inhibition of the hyperalgesia produced by activation of TRPA1-expressing afferents. However, icilin is also a TRPA1 agonist (Story et al., 2003), and its pharmacology is consistent with that of a partial agonist (S. Bevan, personal communication). It could therefore inhibit the effects of cinnamaldehyde by acting as a partial agonist at TRPA1 and thus a competitive antagonist of cinnamaldehyde. This possibility cannot be ruled out as an explanation.

Figure 4.7 TRPA1 activation is hyperalgesic in naïve and CCI animals

a, b) Behavioural data show the effect of intrathecal injection of cinnamaldehyde (75 nmol) in (a) naïve and (b) CCI animals. In naïve and CCI animals, cinnamaldehyde produced behavioural reflex sensitisation (both ipsilateral and contralateral to CCI), as shown by lowered threshold to thermal and mechanical stimuli.

Data show mean ± SEM (from an n of 6 animals) PWL (s) to a noxious thermal stimulus and PWT (mN/mm^2) to graded mechanical stimuli.

○: ipsilateral paw values, ■: naïve/contralateral paw values. * indicates significant ($p<0.05$) ipsilateral-contralateral differences, as measured by Student's t-test for thermal data, and Wilcoxon test for mechanical data; † indicates a significant ($p<0.05$) difference from pre-drug baseline values, as measured by One-Way RM ANOVA followed by Dunnett's post-hoc test for thermal data, and Friedman ANOVA on Ranks followed by Dunn's post-hoc test for mechanical data.

c) Bar chart displays data from ipsilateral paw in CCI animals, which shows that co-injection of the non-selective TRPA1 blocker ruthenium red (0.25 nmol) inhibits thermal and mechanical sensitisation produced by intrathecal cinnamaldehyde (75 nmol), but not the thermal and mechanical analgesia produced by intrathecal icilin (10 nmol). Values are ipsilateral response threshold as a percentage of baseline value, mean ± SEM. Statistically significant changes in thermal/mechanical threshold values due to cinnamaldehyde or icilin (†) compared to pre-drug baseline are indicated, ($p<0.05$, One-Way ANOVA for thermal, Friedman ANOVA on ranks for mechanical) and statistically significant reversal of the effect of cinnamaldehyde in the presence of ruthenium red is indicated (††, $p<0.05$).

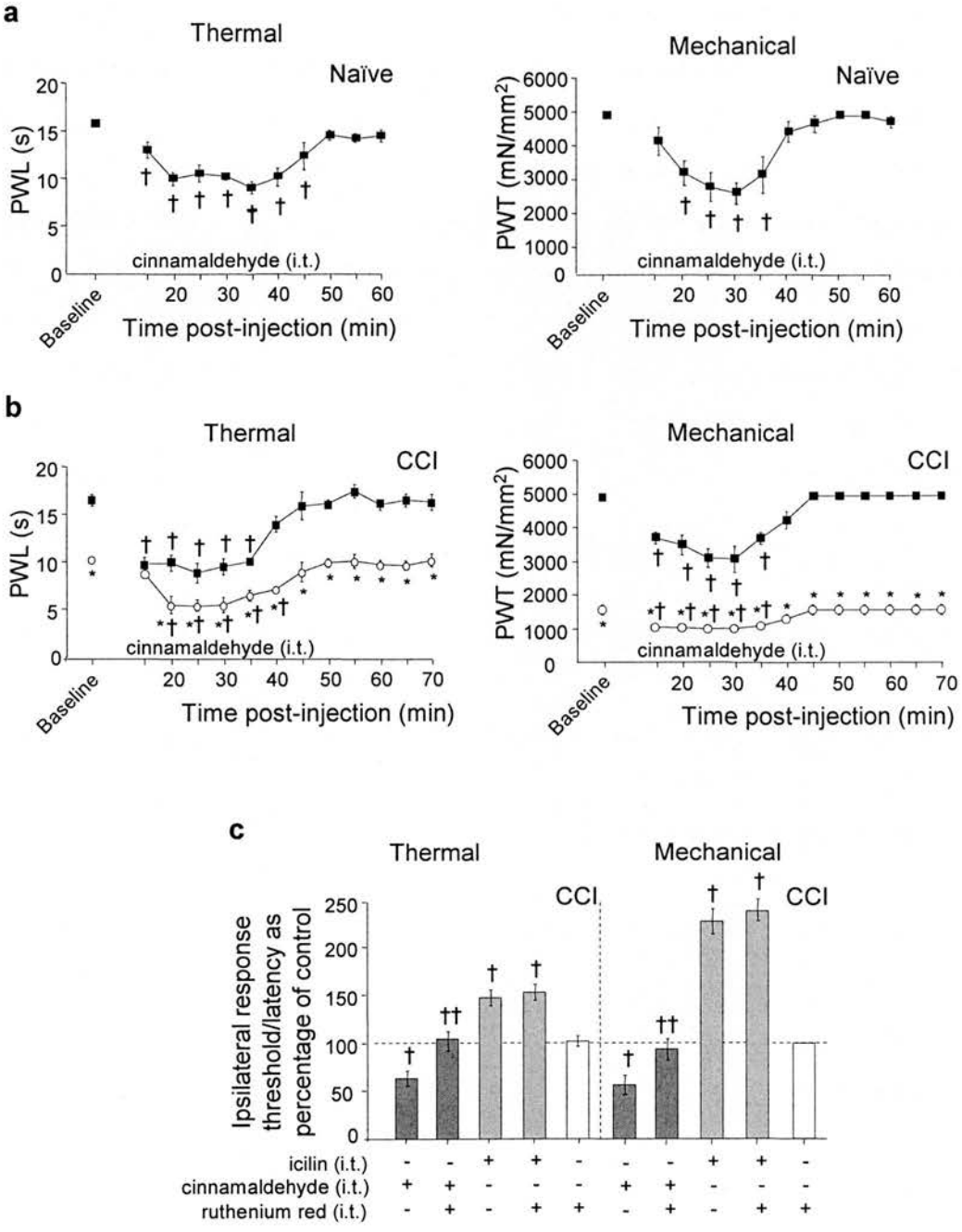

a
Thermal
Naïve
PWL (s)
cinnamaldehyde (i.t.)
Baseline
Time post-injection (min)
Mechanical
Naïve
PWT (mN/mm2)
cinnamaldehyde (i.t.)
Baseline
Time post-injection (min)
b
Thermal
CCI
PWL (s)
cinnamaldehyde (i.t.)
Baseline
Time post-injection (min)
Mechanical
CCI
PWT (mN/mm2)
cinnamaldehyde (i.t.)
Baseline
Time post-injection (min)
c
Thermal
Mechanical
CCI
CCI
Ipsilateral response threshold/latency as percentage of control
icilin (i.t.)
cinnamaldehyde (i.t.)
ruthenium red (i.t.)

Figure 4.7 continued:

d) Intrathecal injection of the TRPA1 agonist diallyl disulphide (DADS; 50 nmol) produces thermal and mechanical behavioural reflex sensitisation in naïve animals.
e) Topical application of cinnamaldehyde (5 min paw immersion in foot-tubes containing 1.5mM cinnamaldehyde at 30 °C) produces thermal and mechanical behavioural reflex sensitisation in naïve animals.
Data show mean ± SEM (from an n of 6 animals) PWL (s) to a noxious thermal stimulus and PWT (mN/mm^2) to graded mechanical stimuli.
■: naïve paw values. † indicates a significant ($p<0.05$) difference from pre-drug baseline values, as measured by One-Way RM ANOVA followed by Dunnett's post-hoc test for thermal data, and Friedman ANOVA on Ranks followed by Dunn's post-hoc test for mechanical data.

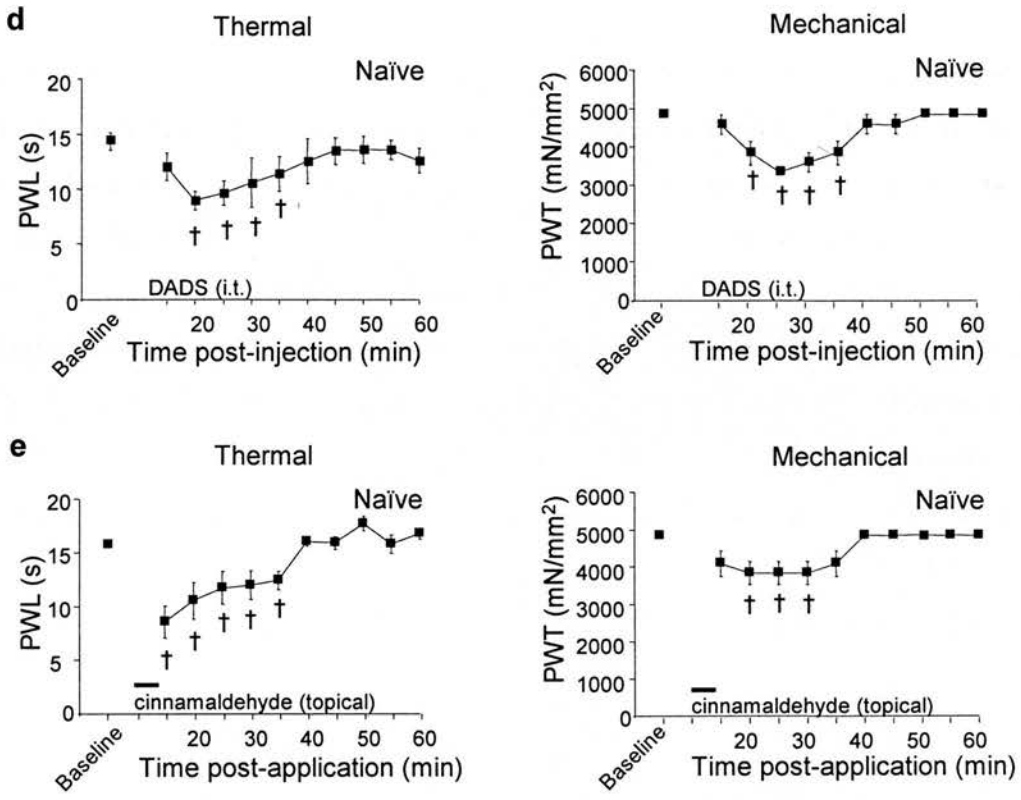

d
Thermal
Naïve
PWL (s)
20
15
10
5
0
DADS (i.t.)
Baseline
20 30 40 50 60
Time post-injection (min)
Mechanical
Naïve
PWT (mN/mm²)
6000
5000
4000
3000
2000
1000
0
DADS (i.t.)
Baseline
20 30 40 50 60
Time post-injection (min)
e
Thermal
Naïve
PWL (s)
20
15
10
5
0
cinnamaldehyde (topical)
Baseline
20 30 40 50 60
Time post-application (min)
Mechanical
Naïve
PWT (mN/mm²)
6000
5000
4000
3000
2000
1000
0
cinnamaldehyde (topical)
Baseline
20 30 40 50 60
Time post-application (min)

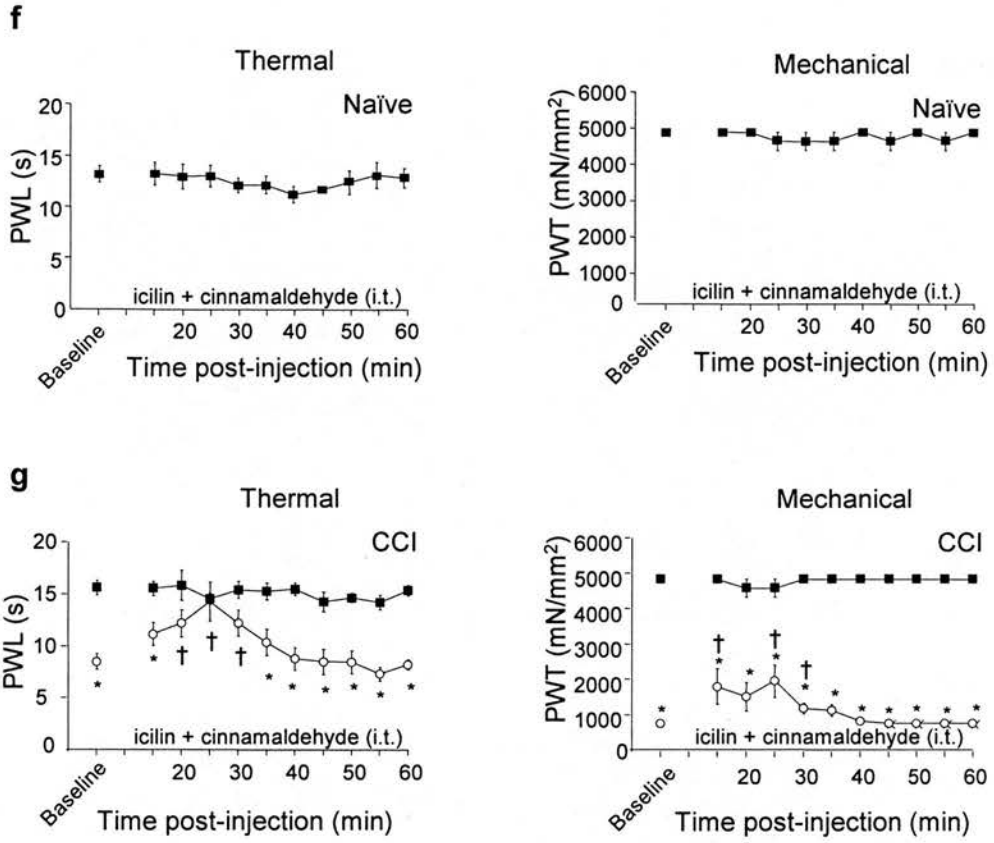
Figure 4.7 continued:

f) Behavioural data show that co-injection of icilin (10 nmol) together with cinnamaldehyde (75 nmol) blocks the sensitisation of thermal and mechanical thresholds produced by cinnamaldehyde in naïve animals, with no net change in reflex responsiveness.

g) Behavioural data show that co-injection of icilin (10 nmol) with cinnamaldehyde (75 nmol) in CCI animals produces an ipsilateral analgesia which reverses the cinnamaldehyde-mediated sensitisation shown above in b).

Data show mean ± SEM (from an n of 6 animals) PWL (s) to a noxious thermal stimulus and PWT (mN/mm^2) to graded mechanical stimuli.

○: ipsilateral paw values, ■: naïve/contralateral paw values. * indicates significant ($p<0.05$) ipsilateral-contralateral differences, as measured by Student's t-test for thermal data, and Wilcoxon test for mechanical data; † indicates a significant ($p<0.05$) difference from pre-drug baseline values, as measured by One-Way RM ANOVA followed by Dunnett's post-hoc test for thermal data, and Friedman ANOVA on Ranks followed by Dunn's post-hoc test for mechanical data.


f
Thermal
Naïve
PWL (s)
20
15
10
5
0
icilin + cinnamaldehyde (i.t.)
Baseline
20 30 40 50 60
Time post-injection (min)
Mechanical
Naïve
PWT (mN/mm²)
6000
5000
4000
3000
2000
1000
0
icilin + cinnamaldehyde (i.t.)
Baseline
20 30 40 50 60
Time post-injection (min)
g
Thermal
CCI
PWL (s)
20
15
10
5
0
icilin + cinnamaldehyde (i.t.)
Baseline
20 30 40 50 60
Time post-injection (min)
Mechanical
CCI
PWT (mN/mm²)
6000
5000
4000
3000
2000
1000
0
icilin + cinnamaldehyde (i.t.)
Baseline
20 30 40 50 60
Time post-injection (min)

4.8 The central mechanism of TRPM8-mediated analgesia involves Group II/III metabotropic glutamate receptors

4.8.1 Rationale

As topical icilin increases activity in fine afferents, and both intrathecal and topical icilin reverse nerve injury-induced sensitisation, centrally-mediated events are likely to be important in icilin-mediated analgesia. Icilin-responsive afferents are expected to release glutamate, so inhibitory glutamate receptors in the dorsal horn of the spinal cord could underlie icilin-induced analgesia. Group II/III mGluRs are antinociceptive in models of inflammatory, neuropathic and acute pain (Chen & Pan, 2005; Fisher & Coderre, 2002; Simmons et al., 2002) and inhibit transmission between primary afferent and spinal cord neurons in sensitised states (Gerber et al, 2000; Neugebauer et al., 2000), and are therefore strong candidates for mediators of inhibition. These receptors are discussed in greater detail in Introduction section 1.8.6 - 8.

4.8.2 Group II/III mGluR antagonists reverse icilin-induced analgesia

To assess whether icilin-analgesia is mediated through Group II/III mGluRs, Group II/III antagonists were co-injected with icilin. I used the selective Group II mGluR antagonist LY 341495 and the selective Group III antagonist UBP 1112. Trials of lower doses of these compounds (0.5 nmol and 2.5 nmol of each compound, in saline vehicle) had no apparent effect on the effect of intrathecal icilin. When the 2.5 nmol dose of LY 341495 was co-injected with icilin, there was an icilin-induced mean percentage reversal of ipsilateral sensitisation over 15 – 30 minutes of 67 ± 6.5% for PWL and 61.9 ± 7.9% for PWT (mean ± SEM). Icilin-induced mean percentage reversal of ipsilateral sensitisation (over 15 – 30 minutes) when co-injected with 2.5 nmol UBP 1112 was 77 ± 7.3% for PWL and 66.8 ± 5.6% for PWT (mean ± SEM). These values, although slightly lowered, are comparable to those for 10 nmol icilin alone (84.7 ± 6.0% for PWL, 76.8 ± 5.3% for PWT). However, higher doses of LY 341495 (5 nmol in saline) and UBP 1112 (10 nmol in saline) each produced a strong, almost complete block of the analgesic effect of 10 nmol icilin (Figure 4.8a,b). Neither LY 341495 nor UBP 1112 had any effects alone in CCI or naïve animals at these doses (10 nmol UBP 1112 in CCI animals is shown as an

example in Figure 4.8d), suggesting that Group II/III mGluRs show little tonic activation but are specifically activated downstream of icilin action. In contrast, co-injection of the μ-opioid receptor antagonist naloxone (25 nmol in saline) had no effect on the analgesic action of icilin (Figure 4.8c), showing that icilin-analgesia is opioid-independent. Matching the profile of icilin, the analgesia produced by intrathecal (-)-menthol (200 nmol, as shown in Figure 4.4b) was reversed by intrathecal LY 341495 and UBP 1112. The mean percentage reversal ± SEM of sensitisation over 20-30 minutes post-injection was 86.1 ± 8.1% for PWL and 80.6 ± 4.2% for PWT with menthol alone, 22.0 ± 6.9% for PWL and 7.1 ± 7.1% for PWT with menthol and LY 341495 (5 nmol), and 9.2 ± 6.9% for PWL and 0.0 ± 0.0% for PWT with menthol and UBP 1112 (10 nmol). As lower doses of LY 341495 and UBP 1112 were ineffective against icilin analgesia, I did not investigate the effect of these doses on menthol.

In order to eliminate the possibility that there are non-specific interactions between the two drugs (icilin plus antagonist) when administered intrathecally, these experiments were performed administering icilin topically, but the mGluR antagonists intrathecally. Figure 4.8e shows that icilin-mediated (80 μM, topically applied in small foot-tubes) reversal of thermal and mechanical sensitisation was prevented by intrathecal LY 341495 (5 nmol) or UBP 1112 (10 nmol). The reversal of mechanical sensitisation produced by skin cooling to 16 °C (Figure 4.3i) was also prevented by intrathecally applied LY 341495 or UBP 1112. The mean percentage reversal of ipsilateral CCI-induced reductions in PWT caused by cooling was 0.0 ± 0.0% in the presence of either drug. Therefore the inhibition of the effect of icilin is still present when the two drugs are administered separately, indicating that the inhibition is not due to non-specific interactions. This result further indicates that the effect of peripheral icilin or cooling is mediated centrally, as it is blocked by centrally administered mGluR antagonists.

4.8.3 Group II/III mGluR agonists mimic the effect of icilin

To assess whether activation of Group II/III mGluRs mimics icilin reversal of neuropathic sensitisation, I intrathecally injected the selective Group II mGluR agonist 2R, 4R-APDC, and the Group III mGluR agonists ACPT-III and AP-4. 2R,

4R-APDC (15 nmol) caused reversal of ipsilateral thermal and mechanical sensitisation, with no effect on contralateral responses, in CCI animals (Figure 4.8f) (mean percentage reversal of ipsilateral-contralateral differences was 72.1 ± 6.4% for thermal and 56.0 ± 10.9% for mechanical). ACPT-III (Figure 4.8g) and AP-4 (Figure 4.8h) (150 nmol each) also reversed thermal sensitisation (by 83.6 ± 6.3% and 60.8 ± 6.7%, respectively), as well as mechanical sensitisation (65.7 ± 11.4% and 60.7 ± 8.0%), again with no effects contralaterally. (All quoted data is mean ± SEM, calculated over 15 – 30 min post-injection). There were no additional behavioural or other physiological effects observed following agonist application, which was a concern, as mGluRs in the central nervous system are implicated in a wide range of functions (Neugebauer, 2002). This is in agreement with previously published results using Group II/III mGluRs in pain models (Chen & Pan, 2005; Fisher et al., 2002; Simmons et al., 2002), which also did not report any additional behavioural effects of such agonists.

Figure 4.8 Icilin-induced analgesia following CCI is prevented by Group II and III mGluR antagonists

a, b, c) Behavioural data show that the analgesic effect of intrathecal icilin (10 nmol) in CCI animals is blocked by co-administration of (a) the Group II mGluR antagonist LY 341495 (5nmol) and (b) the Group III mGluR antagonist UBP 1112 (10 nmol) but not (c) the opioid antagonist naloxone (25 nmol). Blockade of icilin's action is indicated by the persistence of ipsilateral-contralateral differences (*) and by the lack of significant drug alteration of baseline.

Data show mean ± SEM (from an n of 6 animals) PWL (s) to a noxious thermal stimulus and PWT (mN/mm^2) to graded mechanical stimuli.

○: ipsilateral paw values, ■: contralateral paw values. * indicates significant ($p<0.05$) ipsilateral-contralateral differences, as measured by Student's t-test for thermal data, and Wilcoxon test for mechanical data; † indicates a significant ($p<0.05$) difference from pre-drug baseline values, as measured by One-Way RM ANOVA followed by Dunnett's post-hoc test for thermal data, and Friedman ANOVA on Ranks followed by Dunn's post-hoc test for mechanical data.

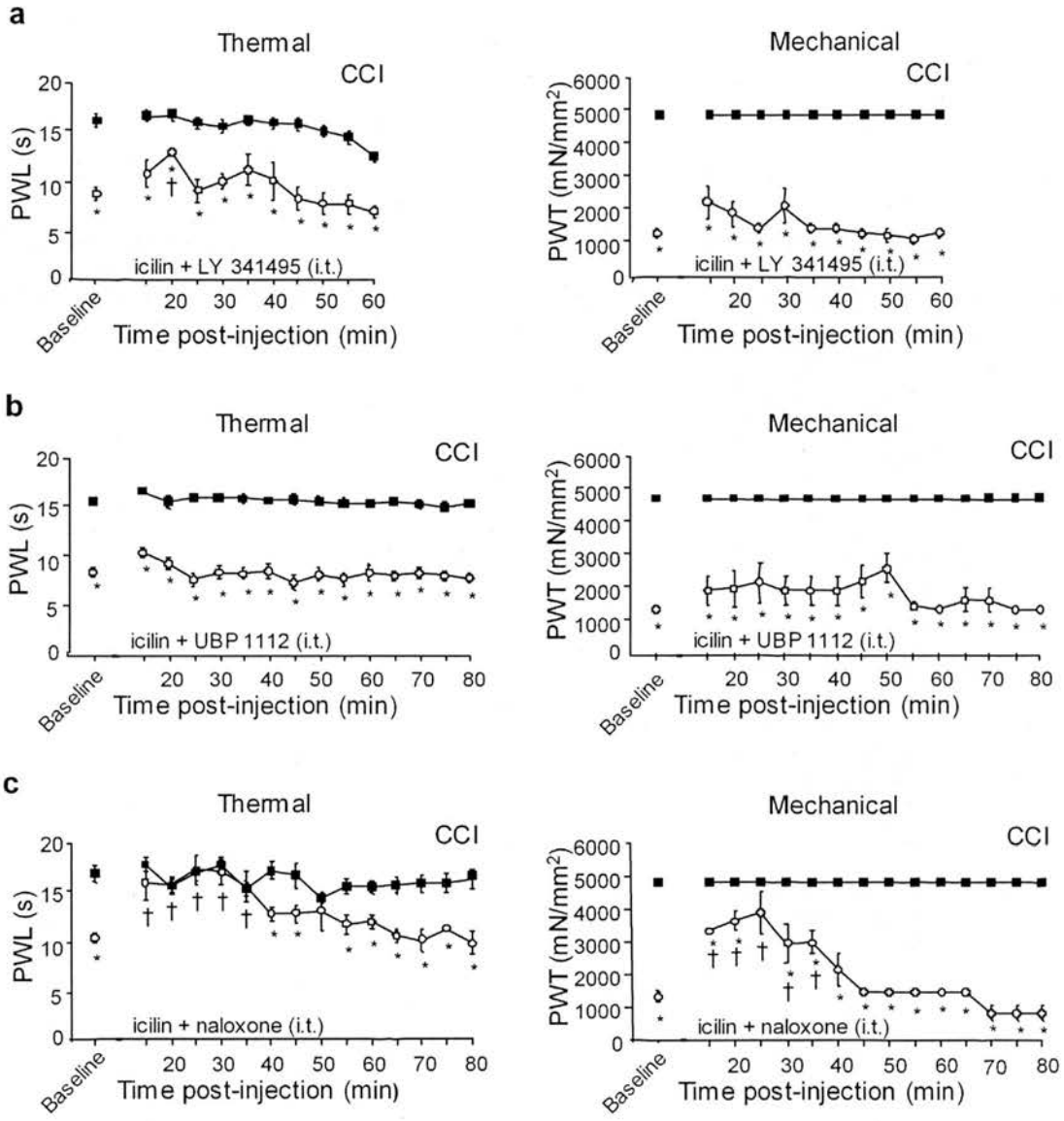

a
Thermal
CCI
PWL (s)
icilin + LY 341495 (i.t.)
Baseline
Time post-injection (min)
Mechanical
CCI
PWT (mN/mm2)
icilin + LY 341495 (i.t.)
Baseline
Time post-injection (min)
b
Thermal
CCI
PWL (s)
icilin + UBP 1112 (i.t.)
Baseline
Time post-injection (min)
Mechanical
CCI
PWT (mN/mm2)
icilin + UBP 1112 (i.t.)
Baseline
Time post-injection (min)
c
Thermal
CCI
PWL (s)
icilin + naloxone (i.t.)
Baseline
Time post-injection (min)
Mechanical
CCI
PWT (mN/mm2)
icilin + naloxone (i.t.)
Baseline
Time post-injection (min)

Figure 4.8 continued:

d) Behavioural data show the effect of the mGluR antagonist UBP 1112 (10nmol) alone administered intrathecally in CCI animals. There is no significant effect of drug upon thermal or mechanical thresholds.

Data show mean ± SEM (from an n of 6 animals) PWL (s) to a noxious thermal stimulus and PWT (mN/mm^2) to graded mechanical stimuli.

○: ipsilateral paw values, ■: contralateral paw values. * indicates significant ($p<0.05$) ipsilateral-contralateral differences, as measured by Student's t-test for thermal data, and Wilcoxon test for mechanical data; † indicates a significant ($p<0.05$) difference from pre-drug baseline values, as measured by One-Way RM ANOVA followed by Dunnett's post-hoc test for thermal data, and Friedman ANOVA on Ranks followed by Dunn's post-hoc test for mechanical data.

e) Bar chart shows the mean percentage reversal of thermal and mechanical ipsilateral sensitisation in CCI animals by topical icilin with concurrent intrathecal injection of the Group II mGluR antagonist LY 341495 (5 nmol) or the Group III mGluR antagonist UBP 1112 (10 nmol) or icilin, LY 341495 or UBP 1112 alone. Values are mean percentage reversal ± SEM, calculated over 15-25 min following paw immersion and intrathecal injection. † indicates significant difference from pre-drug baseline, as measured by One-Way RM ANOVA followed a by Dunnett's post-hoc test for thermal data, and Friedman ANOVA on Ranks followed by Dunn's post-hoc test for mechanical data.

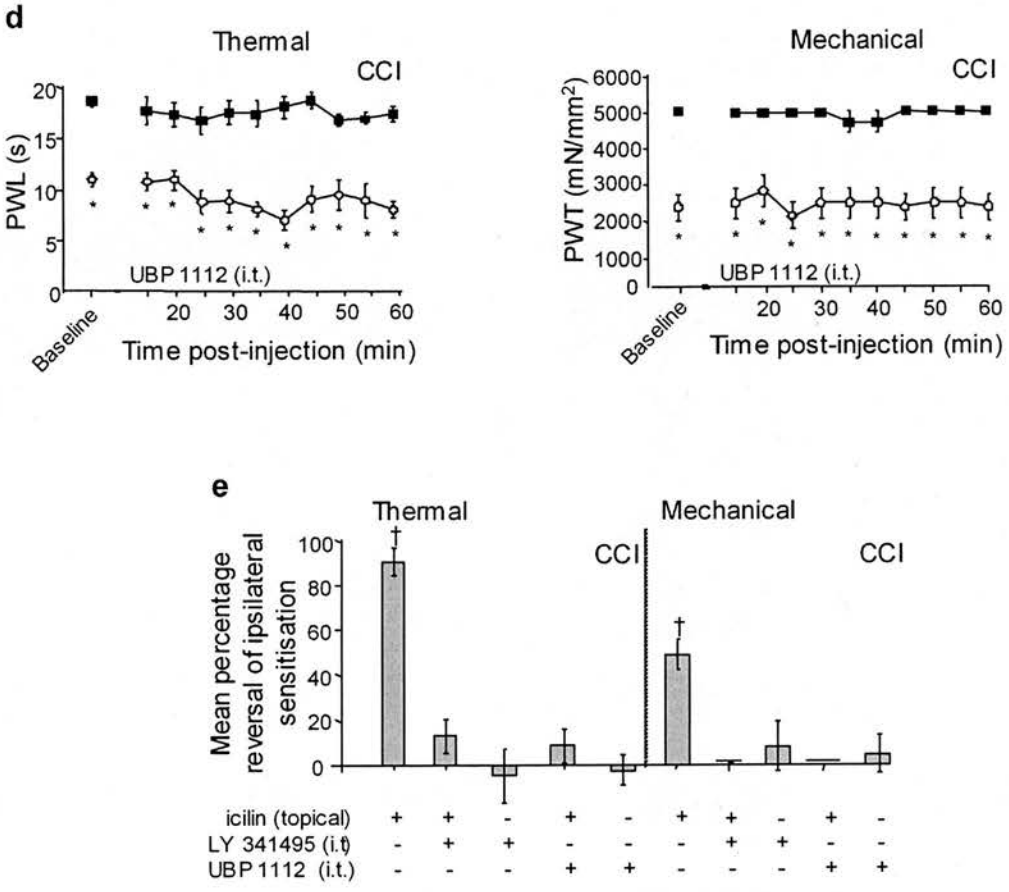
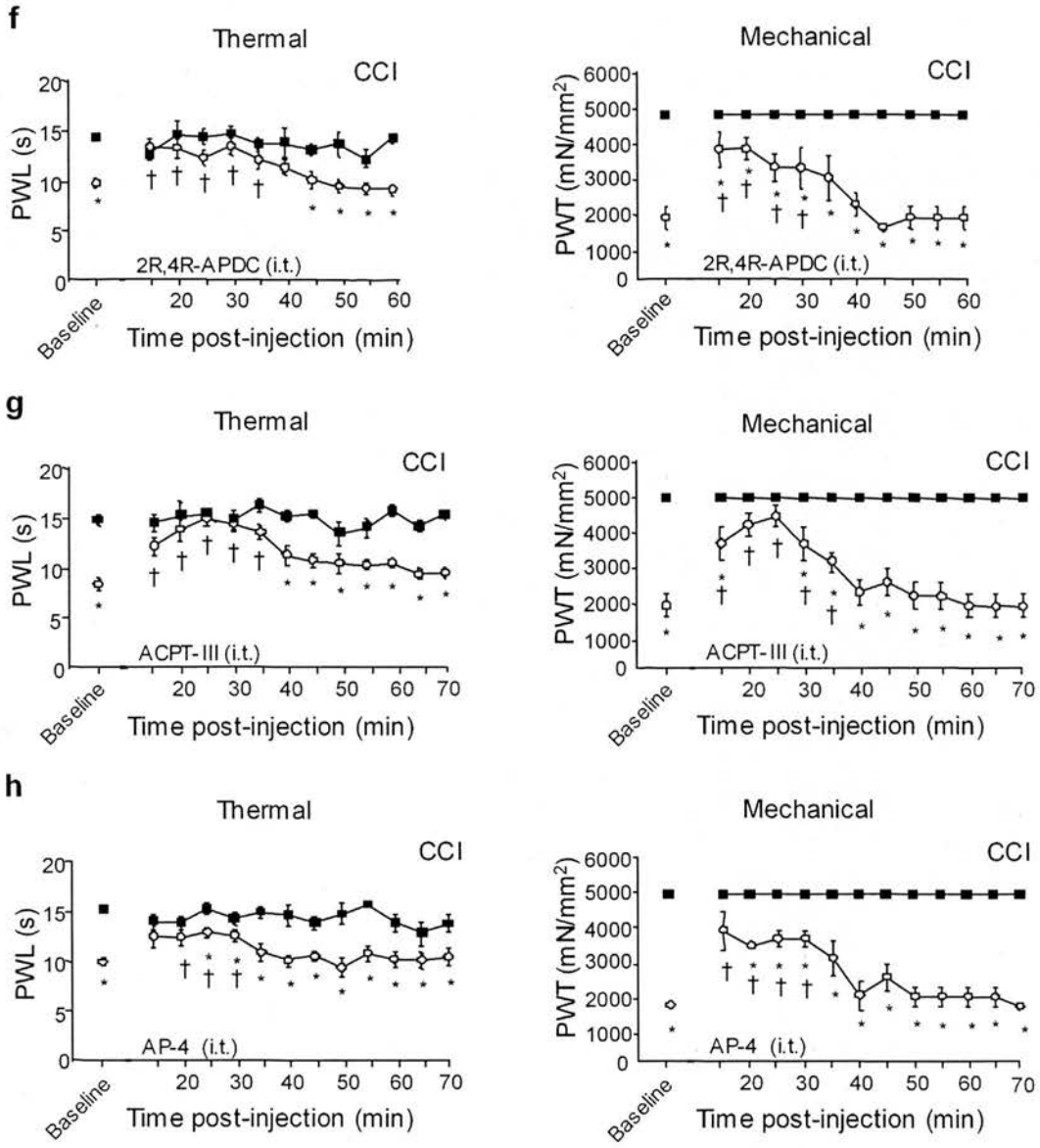

d
Thermal
CCI
PWL (s)
UBP 1112 (i.t.)
Baseline
Time post-injection (min)
Mechanical
CCI
PWT (mN/mm2)
UBP 1112 (i.t.)
Baseline
Time post-injection (min)
e
Thermal
Mechanical
CCI
CCI
Mean percentage reversal of ipsilateral sensitisation
icilin (topical)
LY 341495 (i.t)
UBP 1112 (i.t.)

Figure 4.8 continued:

f, g, h) Behavioural data show that intrathecal administration of the Group II mGluR agonist 2R,4R-APDC (15nmol), and the Group III mGluR agonists ACPT-III (150 nmol) and L-AP4 (150 nmol) in CCI animals produces a reversal of ipsilateral sensitisation, similar to the effect of intrathecal icilin, with no effects on contralateral paw values.

Data show mean ± SEM (from an n of 6 animals) PWL (s) to a noxious thermal stimulus and PWT (mN/mm^2) to graded mechanical stimuli.

○: ipsilateral paw values, ■: contralateral paw values. * indicates significant ($p<0.05$) ipsilateral-contralateral differences, as measured by Student's t-test for thermal data, and Wilcoxon test for mechanical data; † indicates a significant ($p<0.05$) difference from pre-drug baseline values, as measured by One-Way RM ANOVA followed by Dunnett's post-hoc test for thermal data, and Friedman ANOVA on Ranks followed by Dunn's post-hoc test for mechanical data.


f
Thermal
CCI
PWL (s)
2R,4R-APDC (i.t.)
Baseline
Time post-injection (min)
Mechanical
CCI
PWT (mN/mm²)
2R,4R-APDC (i.t.)
Baseline
Time post-injection (min)
g
Thermal
CCI
PWL (s)
ACPT-III (i.t.)
Baseline
Time post-injection (min)
Mechanical
CCI
PWT (mN/mm²)
ACPT-III (i.t.)
Baseline
Time post-injection (min)
h
Thermal
CCI
PWL (s)
AP-4 (i.t.)
Baseline
Time post-injection (min)
Mechanical
CCI
PWT (mN/mm²)
AP-4 (i.t.)
Baseline
Time post-injection (min)

4.9 Icilin-inhibition and mGluR-dependency are apparent at the level of dorsal horn neurons

To confirm the analgesic effect of icilin at the level of single spinal cord neurons, in vivo extracellular recordings were made of large LI and LIII/IV neurons in CCI animals. Laminar location was assessed based on depth of electrode penetration and previous results which used dye ejection to identify the lamina reached by different depths (Moss et al., 2002). Receptive fields were identified by innocuous brush/stroking stimulus, and neurons were identified as WDR neurons by response to both innocuous brush and noxious pinch stimulus. Neurons were then stimulated by a motorised rotating brush applied to the receptive field area. This stimulus is normally innocuous, but would be expected to be noxious ipsilateral to CCI, due to the mechanical allodynia associated with sensitisation, and therefore would be expected to be attenuated by icilin.

Icilin was administered at the same concentration (200 μM) and in the same manner (via a drug-soaked gauze patch applied to part of the peripheral receptive field of the neuron) as in saphenous afferent recording experiments. Topical administration of icilin, but not vehicle (0.2% DMF in water), to the peripheral receptive field area ipsilateral to CCI caused inhibition of neuronal responses to motorised rotating brush (Figure 4.9a,b). In the 8 neurons out of 12 which were affected by icilin (2 in lamina I and 6 in laminae III/IV) brush-induced responses were reduced to 37.4 ± 5.5% of baseline firing frequency, ($p<0.001$, ANOVA on Ranks). The mean time until peak icilin inhibition was approximately 4.5 minutes, which is similar to the results obtained from afferent recordings and from behavioural tests with topical icilin. Contralateral neurons were unaffected by topical icilin, with a mean firing frequency of 111.9 ± 8.9% of baseline ($p>0.05$, $n=6$), shown in Figure 4.9c.

As an example of one of the Group II/III mGluR antagonists, UBP 1112 was ionophoresed in the vicinity of recorded dorsal horn neurons at currents of 20-60 nA. UBP 1112 (200 μM in saline, the same concentration as that applied intrathecally in behavioural tests) reversed the effect of icilin on 7 of the 8 icilin-inhibited ipsilateral neurons; the brush-induced firing rate reverted to 80.2 ± 9.3% of control values

(Figure 4.9b), and, on removal of the ionophoresis current, icilin-mediated inhibition was restored. Neither UBP 1112 nor saline current control (20-60 nA) had any effect alone. These results are summarised in Table 4.5. Ionophoresis of UBP 1112 was not investigated on contralateral neurons due to the lack of icilin effect on these neurons.

The primary aim of these experiments was to identify an inhibitory effect of icilin and examine whether UBP 1112 would reverse this, rather than to examine the timecourse of the icilin effect, and icilin was applied for the duration of the experiments by means of an icilin-soaked gauze patch, so it is impossible to conclude how long the effect of icilin would last independent of further application. Nevertheless, there was clearly no desensitisation to the effect of icilin as, in the one neuron that was inhibited by icilin but showed no effect of UBP 1112, the effect of icilin was apparent for at least 18 minutes (when the experiment was terminated). Further, in those neurons where UBP 1112 did exert an effect, icilin-mediated inhibition was restored on removal of the ionophoresis current, which was up to 13 minutes after initial application.

These results at the level of single spinal cord neurons mirror behavioural results, demonstrating that icilin has an inhibitory effect which is specific to sensitised pain states and which is mediated through inhibitory metabotropic glutamate receptors.

	% of baseline brush-evoked activity following topical vehicle application	% of baseline brush-evoked activity following topical icilin application	% of baseline brush-evoked activity following icilin application with ionophoresed UBP1112 (20-60 nA)	% of baseline brush-evoked activity following icilin application with ionophoresed saline (20-60 nA)	Average time for peak icilin effect (minutes)
Icilin-responsive ipsilateral dorsal horn neurons (n=8)	91.8 ± 7.9	37.4 ± 5.5 *	80.2 ± 9.3 †	52.0 ± 8.9 *	4.5 ± 1.1
Icilin-unresponsive ipsilateral dorsal horn neurons (n=4)	98.7 ± 8.2	95.8 ± 7.2	-	-	-
Contralateral neurons (n=6)	111.7 ± 10.2	111.9 ± 8.9	-	-	-

Table 4.5 Summary of dorsal horn neuron recording following topical icilin/vehicle and ionophoresis of UBP 1112.

* denotes significant difference from baseline brush-evoked activity ($p<0.05$, ANOVA on Ranks)

† denotes significant effect of ionophoresed UBP 1112 on icilin-inhibition of brush-evoked activity ($p<0.05$ ANOVA on Ranks)

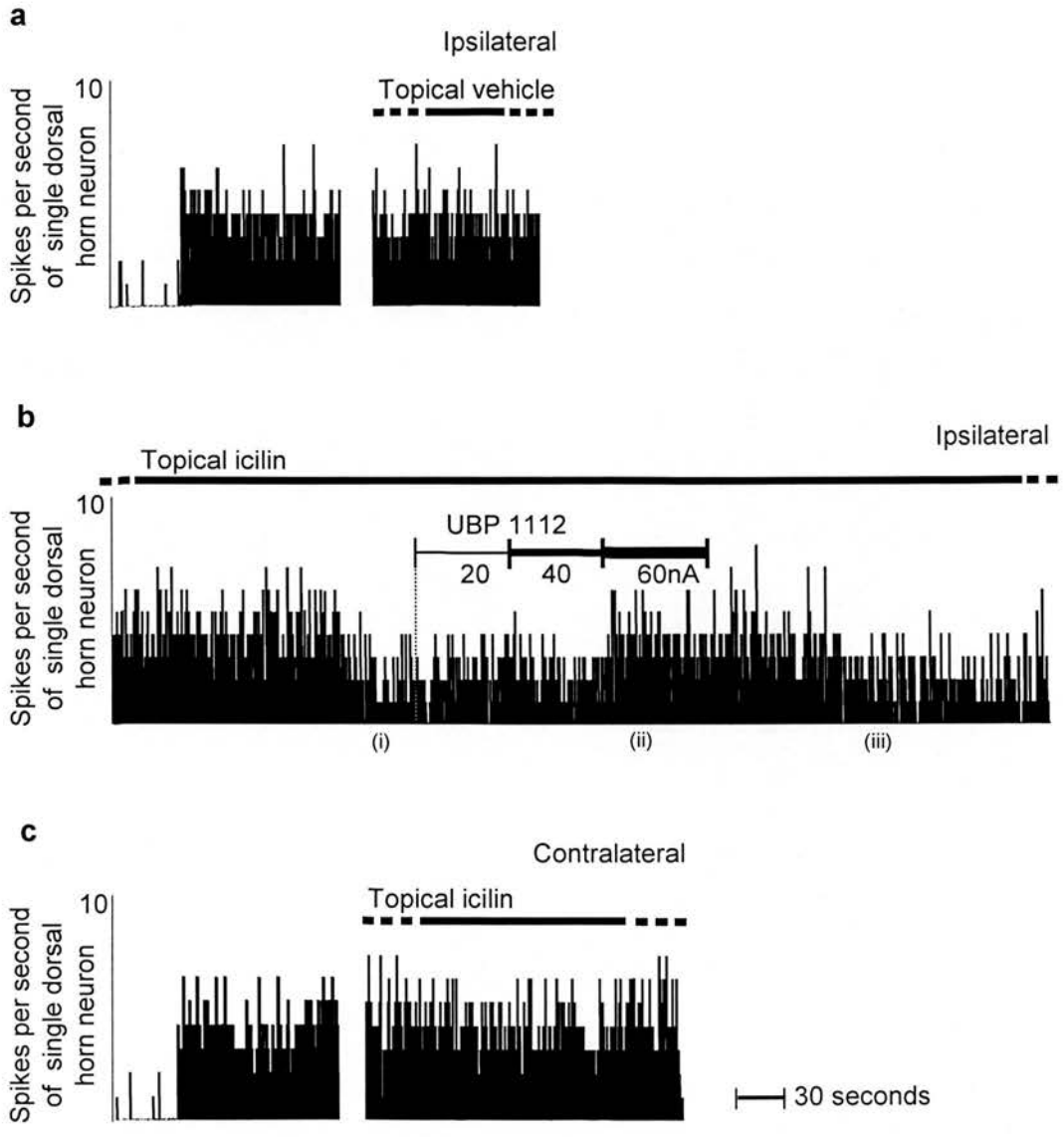
Figure 4.9 Icilin inhibits the sensitised response of ipsilateral dorsal horn neurons in neuropathic animals, which is reversed by a Group III mGluR antagonist

Figure shows responses of single dorsal horn neurons ipsilateral (a, b) and contralateral (c) to CCI, activated by motorised rotating brush stimulus to the receptive field. Neuronal firing is displayed as action potentials per second plotted against time.

a): Vehicle (0.2% DMF in saline, 30°C) topically applied to the receptive field area of the hindpaw (adjacent to motorised brush stimulus) had no effect on ipsilateral dorsal horn neuronal firing.

b): Typical example of the effect of icilin (200 μM in saline, 30°C) topically applied to the receptive field area of the hindpaw (adjacent to motorised brush stimulus) on an ipsilateral dorsal horn neuron. (i): Brush-evoked firing in neurons ipsilateral to nerve injury (reflecting mechanical allodynia) was inhibited by topically applied icilin, (ii): this effect was reversed by ionophoresis of UBP 1112 (200 μM) at currents of 20-60 nA; (iii): recovery was observed following removal of the UBP 1112 ejection current. Similar effects were observed in 8 out of 12 ipsilateral neurons. The icilin-induced reduction in mean firing frequency of the affected dorsal horn neurons was statistically significant ($p<0.001$, ANOVA on Ranks) as was its reversal in the presence of UBP 1112 ($p<0.05$).

c): Typical example of the effect of icilin (200 μM in saline, 30°C) topically applied to the receptive field area of the hindpaw (adjacent to motorised brush stimulus) on a contralateral dorsal horn neuron. There is no apparent change in firing frequency.


a
Ipsilateral
Topical vehicle
10
Spikes per second of single dorsal horn neuron
b
Topical icilin
Ipsilateral
10
UBP 1112
20
40
60nA
Spikes per second of single dorsal horn neuron
(i)
(ii)
(iii)
c
Contralateral
Topical icilin
10
Spikes per second of single dorsal horn neuron
30 seconds

4.10 Possibility of a postsynaptic mechanism of action by icilin

As some Group II/III mGluRs may be postsynaptic, I investigated whether icilin could reverse the additional sensitisation of behavioural reflex responsiveness caused by intrathecally applied NMDA in naïve and CCI animals.

Initial experiments injecting NMDA (30 nmol in saline) showed no effect on behavioural thresholds (mean percentage change in baseline was -3.5 ± 4.2% for PWL and 0.0 ± 0.0% for PWT, values from 15 – 30 minutes post-injection, mean ± SEM, n of 6 animals). However, co-injection of NMDA (30 nmol) with the drug ACPC (6 nmol in saline), which acts as a co-agonist at the glycine site of the NMDA receptor did elicit thermal and mechanical sensitisation in naïve animals (Figure 4.10a). This model of sensitisation was then used to investigate the effect of icilin.

NMDA and ACPC-induced sensitisation was blocked by co-injection of icilin (10 nmol, Figure 4.10b), demonstrating that icilin is active against NMDA-induced hypersensitivity. This was further investigated in CCI animals, where a lower dose of NMDA (3.75 nmol) and ACPC (0.75 nmol) was used to produce an additional ipsilateral sensitisation without affecting contralateral thresholds (Figure 4.10c). Icilin (10 nmol) clearly attenuated the additional ipsilateral sensitisation induced by the NMDA and ACPC (Figure 4.10d), and also went some way to reversing the CCI-induced sensitisation, similar to its effects when injected alone. The blockade of NMDA-mediated hypersensitivity suggests that a component of the central events elicited by icilin may therefore be postsynaptic, although it is important to note that functional NMDA receptors are also present on afferent terminals (Bardoni et al., 2004; Liu et al., 1994).

Figure 4.10 Icilin inhibits the behavioural sensitisation produced by intrathecal NMDA

a, b) Behavioural data show that in naïve animals (a) intrathecal injection of NMDA (30 nmol) together with its glycine site co-agonist ACPC (6 nmol) produce sensitisation of behavioural reflexes to thermal and mechanical stimuli, and that (b) this effect is blocked by co-injection of icilin (10 nmol), with no net effect on thresholds to stimuli.

Data show mean ± SEM (from an n of 6 animals) PWL (s) to a noxious thermal stimulus and PWT (mN/mm^2) to graded mechanical stimuli.

■: naïve paw values. † indicates a significant ($p<0.05$) difference from pre-drug baseline values, as measured by One-Way RM ANOVA followed by Dunnett's post-hoc test for thermal data, and Friedman ANOVA on Ranks followed by Dunn's post-hoc test for mechanical data.

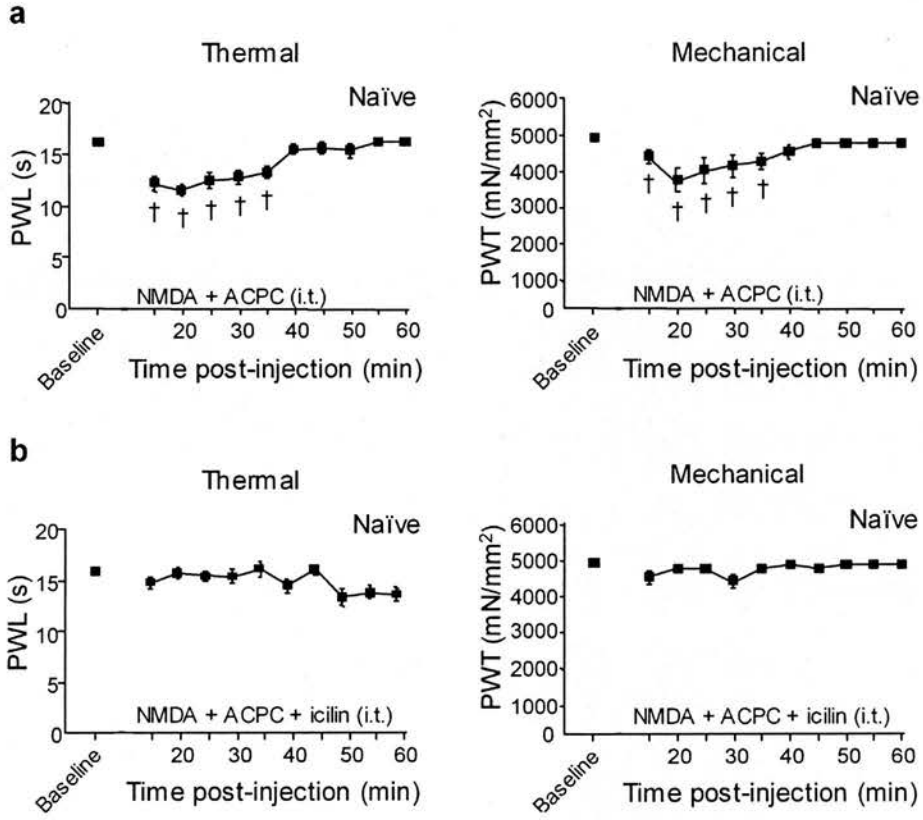
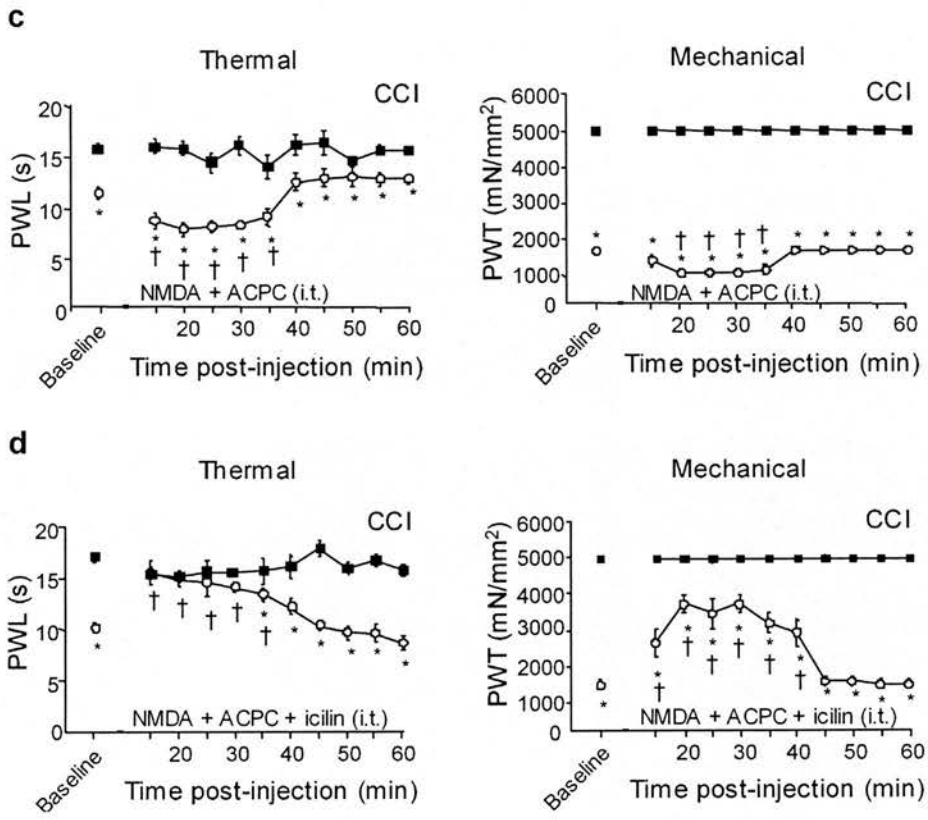

a
Thermal
Naïve
PWL (s)
NMDA + ACPC (i.t.)
Baseline
Time post-injection (min)
Mechanical
Naïve
PWT (mN/mm²)
NMDA + ACPC (i.t.)
Baseline
Time post-injection (min)
b
Thermal
Naïve
PWL (s)
NMDA + ACPC + icilin (i.t.)
Baseline
Time post-injection (min)
Mechanical
Naïve
PWT (mN/mm²)
NMDA + ACPC + icilin (i.t.)
Baseline
Time post-injection (min)

Figure 4.10 continued:

c, d) Behavioural data show that in CCI animals (c) intrathecal injection of NMDA (3.75 nmol) together with its glycine site co-agonist ACPC (0.75 nmol) produce a further sensitisation of ipsilateral behavioural reflexes to thermal stimuli and a small but significant sensitisation of reflexes to mechanical stimuli, with no discernible effect on contralateral paw values, and that (d) co-injection of icilin (10 nmol) produces an ipsilateral analgesia, which cancels out the NMDA-induced sensitisation.

Data show mean ± SEM (from an n of 6 animals) PWL (s) to a noxious thermal stimulus and PWT (mN/mm^2) to graded mechanical stimuli.

○: ipsilateral paw values, ■: contralateral paw values. * indicates significant ($p<0.05$) ipsilateral-contralateral differences, as measured by Student's t-test for thermal data, and Wilcoxon test for mechanical data; † indicates a significant ($p<0.05$) difference from pre-drug baseline values, as measured by One-Way RM ANOVA followed by Dunnett's post-hoc test for thermal data, and Friedman ANOVA on Ranks followed by Dunn's post-hoc test for mechanical data.


c
Thermal
CCI
PWL (s)
NMDA + ACPC (i.t.)
Baseline
Time post-injection (min)
Mechanical
CCI
PWT (mN/mm2)
NMDA + ACPC (i.t.)
Baseline
Time post-injection (min)
d
Thermal
CCI
PWL (s)
NMDA + ACPC + icilin (i.t.)
Baseline
Time post-injection (min)
Mechanical
CCI
PWT (mN/mm2)
NMDA + ACPC + icilin (i.t.)
Baseline
Time post-injection (min)

4.11 Protein expression of Group II/III metabotropic glutamate receptors

4.11.1 Western blots show no change in levels of Group II/III mGluRs

Alterations in mGluR expression could lead to increased efficacy of the TRPM8-mGluR axis, and might underlie the effectiveness of icilin in CCI. To investigate this mGluR expression was analysed by Western blots. I examined the expression of mGluR7, mGluR4 (Group III mGluRs), and of mGluR2/3.

Immunoblotting with an antibody against mGluR7 showed a band at approximately 97 kDa (the predicted molecular weight of mGluR7; Yao et al., 2005). This band showed no change in expression in DRG or spinal cord whole lysates compared between ipsilateral or contralateral CCI tissue and compared with naïve tissue (Figure 4.11a).

Immunoblotting with an antibody against mGluR4 showed a single, albeit faint, band at ~100 kDa in DRG samples, consistent with the predicted molecular weight of mGluR4 (Yao et al., 2005). There was no signal in spinal cord tissue (blot not shown) and expression was unchanged in ipsilateral or contralateral DRG (Figure 4.11b).

Immunoblotting with an antibody against mGluR2/3 showed a single band at ~100 kDa, consistent with the predicted molecular weight of these subtypes (predicted molecular weight 100-110, Yao et al., 2005). There was no alteration in the staining pattern in DRG or spinal cord samples following CCI (Figure 4.11c). As mGluR3 in particular is found located postsynaptically (Tamaru et al., 2003), spinal cord samples were also probed with an antibody specific to mGluR3. This similarly gave a single band at ~100 kDa, with no change in staining intensity following CCI. However, mGluR3 appeared significantly upregulated ipsilateral to CFA treatment (Figure 4.11d), although this was not pursued further, as the CCI was our primary model of interest. This confirms previous reports that mGluR3 increases in models of chronic inflammatory pain (Boxall et al., 1998; Dolan et al., 2003). All blots used GAPDH as a loading control. Therefore alterations in mGluR expression do not seem to occur following CCI. This is consistent with the suggestion that such alterations do not underlie the increased efficacy of icilin in this pain state.

4.11.2 Immunohistochemistry shows mGluR7 in DRG

As an example of an inhibitory glutamate receptor, the expression of mGluR7 was analysed by immunohistochemistry. Immunostaining of DRG for mGluR7 revealed the presence of mGluR7 in the majority of peripherin-positive cells, with no alteration following CCI, Figure 4.11e. mGluR7 was found in 70 ± 1.3% peripherin-positive cells ipsilateral to CCI, 68 ± 2.2% contralateral to CCI and 73 ± 1.8% in naïve animals (no significant differences, $p>0.05$, n=3 CCIs, 3 naïve animals). This confirms Western blots and agrees with previously published results (Li et al., 1996), and suggests that mGluR7 is expressed by a large number of putative nociceptive afferents and could therefore mediate icilin-induced inhibition of nociceptors via a presynaptic location on afferent terminals.

Figure 4.11: There is no alteration in expression of Group II/III mGluRs in CCI animals

a) Immunoblots of DRG and spinal cord whole lysate probed for mGluR7 show protein running at ~ 97 kDa, together with GAPDH loading control at approximately 36 kDa. There is no discernible difference in mGluR7 band intensity in DRG or spinal cord between ipsilateral and contralateral and naïve samples.

b) Immunoblot of DRG whole lysate probed for mGluR4 shows protein running at ~100 kDa, together with GAPDH loading control at approximately 36 kDa. There is no discernible difference in mGluR4 band intensity in DRG between ipsilateral and contralateral samples.

c) Immunoblots of DRG and spinal cord whole lysate probed with an antibody which detects mGluR2 and mGluR3 show protein running at ~100 kDa, together with GAPDH loading control at approximately 36 kDa. There is no discernible difference in mGluR2/3 band intensity in DRG or spinal cord between ipsilateral and contralateral and naïve samples.

d) Immunoblots of spinal cord whole lysate probed for mGluR3 show protein running at ~100 kDa, together with GAPDH loading control at approximately 36 kDa. There is no discernible difference in mGluR3 band intensity in CCI spinal cord between ipsilateral and contralateral and naïve samples. However, in spinal cord whole lysate from CFA-treated animals, there is a significant increase in staining ipsilateral to CFA treatment, with no change in saline-treated animals.

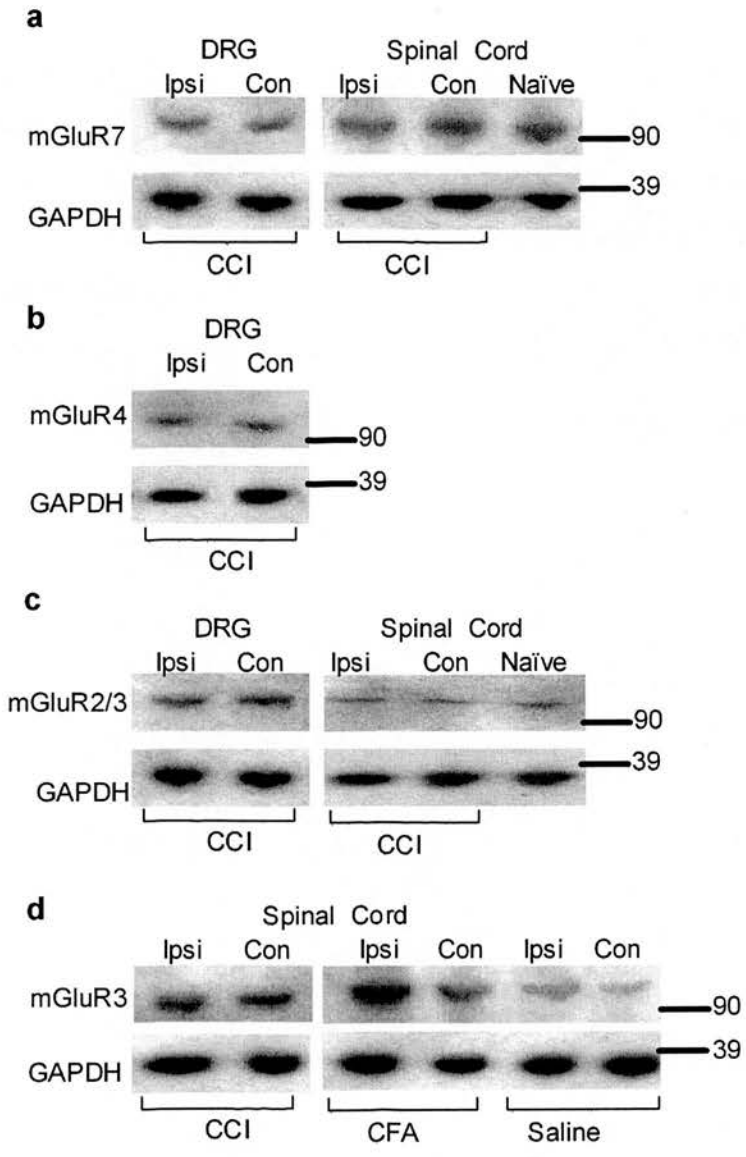
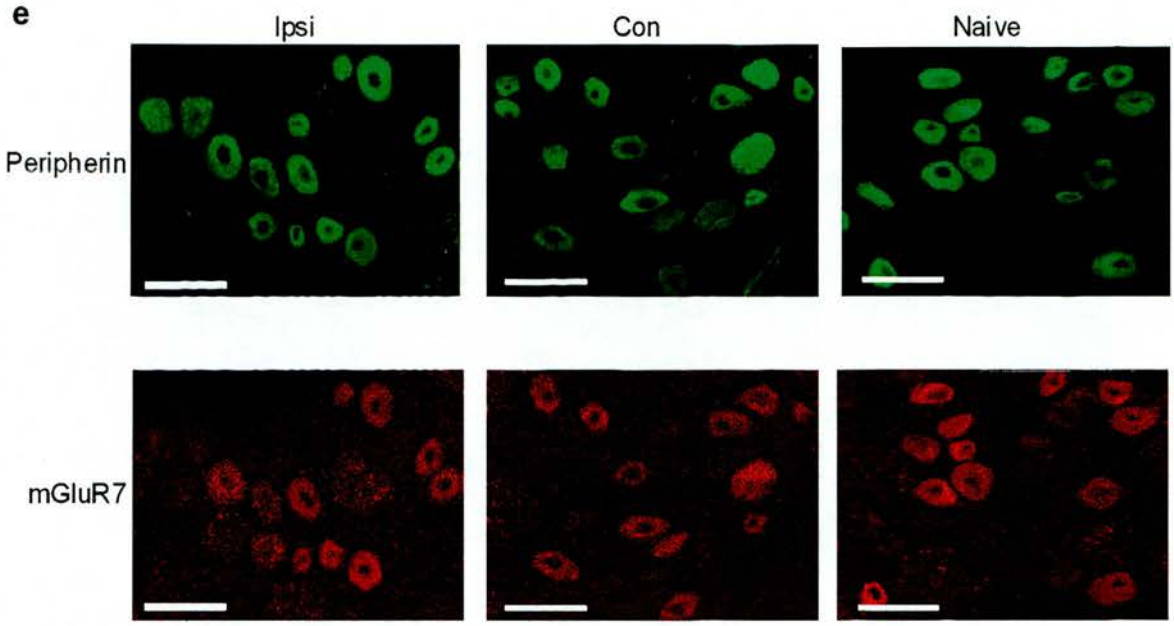

a
DRG
Spinal Cord
Ipsi
Con
Ipsi
Con
Naïve
mGluR7
90
GAPDH
39
CCI
CCI
b
DRG
Ipsi
Con
mGluR4
90
GAPDH
39
CCI
c
DRG
Spinal Cord
Ipsi
Con
Ipsi
Con
Naïve
mGluR2/3
90
GAPDH
39
CCI
CCI
d
Spinal Cord
Ipsi
Con
Ipsi
Con
Ipsi
Con
mGluR3
90
GAPDH
39
CCI
CFA
Saline

Figure 4.11 continued:

e) shows DRG sections ipsilateral (ipsi) or contralateral (con) to CCI and from naive animals, immunostained for mGluR7 (red) and the C-fibre marker peripherin (green), visualised under a confocal microscope. mGluR7 is predominantly expressed in small, peripherin-positive cell bodies and there is no discernible difference in expression of mGluR7 between ipsilateral, contralateral and naïve sections. Scale bar is 50 μm.


e
Ipsi
Con
Naive
Peripherin
mGluR7

Chapter 5: Discussion

5.1 Review of animal models of chronic pain states used in this study

This study utilised several models of chronic pain, which produced changes in the sensitivity of behavioural reflexes. The principal model used was the CCI model of neuropathic pain, which produced robust and consistent thermal hyperalgesia, and mechanical and cold allodynia, ipsilateral to injury, which were maximal between post-operative days 9 to 17. This model has been well-studied, and these observations are in agreement with results reported by other groups (Bennett & Xie, 1988; Kim et al., 1997). Although contralateral behavioural changes have been observed in other models of neuropathy – the PNL (Seltzer et al., 1990) and the SNI (Erichsen & Blackburn-Munro, 2002) models - in my use of the CCI model, I never observed significant effects on contralateral threshold, suggesting that if the CCI model does produce changes in contralateral neuronal processing, they are not strong enough to sustain behavioural changes. Therefore the difference between contralateral and ipsilateral behavioural thresholds, which was used in drug tests as a measure of sensitisation, does seem to be a valid measurement, as the contralateral threshold never differed significantly from the naïve, pre-surgery baseline in the CCI model in the present study.

Alternative surgical neuropathic models, such as the PNL, SNI and SNL models, were not investigated. An investigation and comparison of multiple models of surgical neuropathy was beyond the scope of this project, and further experiments investigated models of other sensitised pain states, such as inflammatory, demyelination-induced and NMDA-induced sensitisation.

A model of demyelination-induced pain, as developed by Wallace et al. (Wallace et al., 2003) was also studied. In this model, topical application of lysolecithin to the sciatic nerve produces focal demyelination of myelinated A fibres, with no axonal loss (Wallace et al., 2003). As a result of this treatment, thermal hyperalgesia and mechanical allodynia develops, and in parallel with behavioural sensitisation, alterations in afferents are observed that resemble those seen in surgical models of

peripheral nerve damage, including heightened spontaneous firing in afferents, decreased expression of the Na^+ channel $Na_v1.8$ and increased expression of $Na_v1.3$, and increased NPY expression (Wallace et al., 2003). In agreement with results reported by Wallace et al., the development of thermal hyperalgesia and mechanical allodynia was observed ipsilateral to injury. In addition, the animals also developed cold hypersensitivity, characterised by withdrawal of the injured paw to a 4°C stimulus, an effect which was not reported by Wallace et al. The response to cold developed over the same timecourse as the thermal and mechanical sensitisation. Cold allodynia - the perception of normally innocuous cold temperatures as noxious - is a common feature of clinical neuropathic pain (Woolf & Mannion, 1999), and has also been reported as a feature of peripheral demyelinating diseases (Carter et al., 1998; Ochoa & Yarnitsky, 1994). Therefore, the demonstration of cold allodynia in the lysolecithin model further supports the use of this model as a model of peripheral demyelination-induced pain states, and supports the idea that this model shows some similarities with neuropathic pain states.

Additional studies utilised a model of inflammatory pain generated by injection of CFA into the hindpaw, in which the development of thermal hyperalgesia and mechanical allodynia was observed ipsilateral to injection, in agreement with others (Raghavendra et al., 2004; Stein et al., 1988). Therefore, in all the models used in this study robust significant changes were seen in behavioural thresholds ipsilateral to injury. This allowed the investigation of the effect of drugs on both the sensitised behavioural responses of the ipsilateral paw, and on the non-sensitised responses of the contralateral limb and naïve animals.

5.2 Background to study of cooling and pain

The phenomenon of analgesia produced by cutaneous cooling has been known for centuries (Adams, 2006; Siegel, 1970; Sprengell, 1735). Clinical trials have demonstrated an analgesic effect of local cooling on a variety of pain states including chronic back pain, post-operative pain, dental pain and muscle injuries (Melzack et al 1980a,b; review by Sauls, 1999), and psychophysical studies show an analgesic effect of local cooling on microelectrode stimulation-induced pain (Bini et al., 1984;

Bugaj, 1975; Saeki et al., 2002). Similarly, substantial evidence supports an analgesic role of menthol and mint oil. Topical mint oil is used in traditional Chinese and European medicine for relief of neuralgia (Wright, 1870; Blumenthal, 1998), and was recently shown to effectively relieve pain in a clinical case study of postherpetic neuralgia (Davies et al., 2002). Topical menthol has been shown to be analgesic in psychophysical studies (Green & McAuliffe, 2000; Gobel et al., 1994), and systemic menthol increased pain thresholds in the mouse hot-plate and acetic acid writhing tests (Galeotti et al., 2002). It has recently been shown that cold and menthol activate a common molecular transduction mechanism, the TRPM8 receptor, which is expressed in subpopulations of sensory neurons (McKemy et al., 2002; McKemy, 2005; Peier et al., 2002b; Reid, 2005). It was hypothesised that, as it is activated by both cold and menthol, TRPM8 might at least partially mediate the analgesic effects of both these stimuli. This led us to investigate the effect of chemical agonists of the TRPM8 receptor in both naïve and sensitised pain models, to see whether TRPM8 activation produced analgesia.

5.3 Analgesia produced by topical application of TRPM8 activators

5.3.1 Analgesia produced by topically applied icilin and menthol

Topical application of icilin, which is a highly potent and efficacious agonist of TRPM8 (Behrendt et al., 2004), to the paws produced a strong and long-lasting reversal of ipsilateral behavioural sensitisation in the CCI model, in a concentration-dependent manner. Similar effects were seen with topical application of menthol. Although neither icilin nor menthol is a completely selective agonist at TRPM8, as is discussed further below in section 5.6, the fact that both icilin and menthol produced analgesia supports the idea that the analgesia is mediated through the common target of these drugs, the TRPM8 receptor. Effects of menthol were seen with several stereoisomers: (-)-menthol, (+)-menthol, and (+)-isomenthol. However, the latter two required higher concentrations in order to exert an effect. Prior to the discovery of TRPM8, it was observed that (-)-menthol exerted a greater potentiation of cold-sensitive currents in sensory neurons than (+)-menthol (Schafer et al., 1986). It has since been shown that (-)-menthol is a more potent activator of TRPM8 than (+)-

menthol, with an EC_{50} of 4.1 ± 1.3 μM, compared with 14.4 ± 1.3 μM for (+)-menthol in similar conditions (Behrendt et al., 2004). Similarly, (-)menthol appears to have a slightly higher potency at TRPM8 than (+)-isomenthol (Bandell et al., 2006). Therefore the observation that (-)-menthol is a more potent analgesic agent than (+)-menthol and (+)-isomenthol is broadly supportive of an analgesic mechanism mediated through the TRPM8 receptor.

These results are exciting because they identify menthol and icilin as novel potential treatments for neuropathic pain conditions. Current treatment options for neuropathic pain are limited (Morley-Forster, 2006; Moulin et al., 2006; Woolf & Mannion, 1999); the most effective medications in current use, which are anti-convulsants and anti-depressants, achieve clinically significant effects in less than 50% of patients (Bridges et al., 2001; Sindrup & Jensen, 1999) and cause deleterious side effects. Therefore the identification of compounds that act as analgesics in neuropathic pain models is potentially very promising for the clinical treatment of neuropathic pain. Icilin and menthol reversed the behavioural sensitisation of the ipsilateral injured hindlimb, but had no effect on contralateral thresholds and similarly had no effect in naïve animals. This ability to reverse sensitisation without affecting normal responses to noxious stimuli is a highly desirable characteristic in an analgesic drug. Very low doses of menthol and icilin were effective in reversing sensitisation, which suggests that such drugs could be used at doses that may be unlikely to produce significant side effects. Furthermore, topical application was effective, indicating a readily usable, non-invasive method of application. Therefore the profile of these drugs shown here is very promising for clinical use.

The effectiveness of menthol in a neuropathic pain model demonstrated here is in line with recent findings in which a case of postherpetic neuralgia (which is a neuropathic condition, although of a distinct aetiology from the surgical models used here) was successfully treated with topical peppermint oil (Davies et al., 2002). The results of this thesis also support the validity of the use of peppermint oil in traditional Chinese and European herbal medicine for treating neuralgia (Wright, 1870; Blumenthal et al., 1998).

5.3.2 Analgesia produced by cold temperatures

In addition to the analgesic effect of TRPM8-activating compounds, physical cooling was also observed to produce an analgesic effect. An analgesic effect was observed on mechanical thresholds of paw immersion in temperatures between 16-20°C. This was fully recovered by 5 minutes later after paw removal. Recordings from a subcutaneous thermistor in anaesthetised animals showed that deep skin temperatures (which presumably reflects more closely the actual temperature of peripheral afferent terminals in the skin) were approximately 0.5°C above bath temperatures after 5 minutes in similar conditions, and therefore could be considered to represent the stimulus temperature.

TRPM8 expressed in heterologous systems has a mean activation threshold of between 19-24°C, and continues to be active at temperatures well below this, with currents saturating at around 10°C (Peier et al., 2002b; McKemy et al., 2002); whereas the presumed TRPM8 current in native sensory neurons has a mean activation threshold of around 28°C, but is still active at temperatures in the range 16 – 20°C (Reid, 2005; Reid et al., 2002). Therefore, it is indeed likely that TRPM8 will be activated by the temperature range in which an analgesic effect was observed. However, other cold-sensitive receptors may also be activated in this temperature range. Alternative cold-sensitive transduction mechanisms, for example an outward K^+ current (Reid & Flonta, 2001b; Viana et al., 2002), and the epithelial sodium channel (Askwith et al., 2001), have been proposed to contribute to cold sensitivity, and it is possible that such mechanisms are also affected by the waterbath temperatures applied. Therefore it is clear that temperature-based stimuli are not as precise in terms of experimental challenges in pain models as chemical agonists for investigating the receptor-based mechanism of cooling-induced analgesia. The difficulty in interpreting temperature-based stimuli with regard to activation of molecular receptors led us to concentrate on chemical means of activating TRPM8 in further experiments.

Immersion temperatures below 14°C elicited withdrawal reflexes of the ipsilateral paw for the duration of the immersion period. This is in agreement with other results describing cold-induced ipsilateral withdrawal reflexes beginning at a threshold of a 15°C (applied temperature) in rats with SNI or SNL nerve injuries (Allchorne et al.,

2005), and with psychophysical experiments that describe noxious cold sensation in humans beginning at temperatures below 15°C (Chen et al., 1996; Chery-Croze, 1983; Wolf & Hardy, 1941). These data suggest that temperatures below ~15°C can activate noxious cold-sensitive neurons and elicit behavioural responses to noxious cold, particularly following neuropathy. Such temperatures may activate additional receptors with lower temperature-activation thresholds than TRPM8. Antisense knockdown of TRPM8 did not alleviate the cold allodynia observed in the SNL model (Katsura et al., 2005), or the CCI model (Results section 4.6.1), indicating that TRPM8 is unlikely to contribute to neuropathy-induced cold allodynia. Other receptor mechanisms may therefore be involved in cold allodynia, such as the putative cold receptor TRPA1. Antisense knockdown of TRPA1 has been shown to suppress the development of cold allodynia following SNL (Katsura et al., 2006; Obata et al., 2005). Moreover, TRPA1 has been reported to respond maximally to temperatures below 17°C (Story et al., 2003), which indicates a range more in line with the temperatures that evoked withdrawal reflexes in my results and those of other groups (Allchorne et al., 2005), although, given the controversy surrounding whether TRPA1 is actually sensitive to cold (as described in Introduction section 1.25.1), the role of TRPA1 in cold allodynia is by no means certain.

5.3.3 Regional specificity of application of TRPM8 activators for anti-sensitisation analgesia

Behavioural experiments demonstrated that it was necessary to apply icilin to the same dermatome as the area of sensitisation in order to have an effect, as application only to the contralateral hindpaw had no effect on ipsilateral paw sensitivity. The relief of pain is therefore specific to the dermatomes over which the drug is applied. This specificity suggests that the drug acts specifically at afferent neurons in the injured paw. It further suggests that the analgesic action is not a generalised, whole-body inhibition of sensitised responses, such as would result from a humoural mechanism, but must be specific to the nervous signals from the area over which the drug is applied.

5.3.4 Hyperalgesia evoked by high doses of icilin

Topical application of high concentrations of icilin (5 mM) caused a generalised increase in sensitivity. This effect was not specific to the sensitised state, affecting CCI animals bilaterally and naïve animals in a similar fashion. The fact that the hyperalgesia is not specific to the sensitised state suggests a separate mechanism to that which mediates analgesia. It is possible that there is spillover of drug onto other, non-TRPM8 receptors, such as TRPA1, activation of which was observed to elicit bilateral hyperalgesia. Alternatively, this hyperalgesic effect could be due to activation of TRPM8-expressing nociceptive afferents. The extent to which TRPM8 is expressed in nociceptive afferents is uncertain. Electrophysiological and imaging experiments in sensory neurons (both cultured and acutely dissociated cells) have shown that some menthol-responsive cells show properties suggestive of nociceptive neurons, such as responsiveness to capsaicin (Babes et al., 2004; McKemy et al., 2002; Xing et al., 2006), sensitivity to ATP, and expression of both TTX-S and TTX-R Na^+ currents (Xing et al., 2006). However, in situ hybridisation and immunohistochemical studies are consistent with a location for TRPM8 in predominantly non-nociceptive neurons, as several studies have observed that TRPM8 is not co-expresssed with TRPV1 (Kobayashi et al., 2005; Peier et al., 2002b) or with CGRP or IB-4 (Peier et al., 2002b), which are markers typically expressed in nociceptive populations. This suggests that TRPM8 is expressed to a large extent in non-nociceptive neurons. However, TRPM8 may also be expressed in polymodal nociceptive neurons, and it is possible that higher doses of icilin activate such neurons to elicit hyperalgesia.

Analgesic effects of icilin were seen at 200-fold lower concentrations than those causing non-specific sensitisation. This suggests that there is quite a large potential therapeutic window, in which analgesia could be produced by low doses of agonist, without the complication of hyperalgesia elicited by higher doses.

5.4 Afferent subpopulations responsive to icilin and menthol

Topical application of icilin (at the low doses used to produce analgesia) to the receptive field activated a subset of saphenous nerve afferents with conduction

velocities in the Aδ/C fibre conduction velocity range, the majority (95%) of which were C fibres. There was no significant effect of icilin on a range of afferents that were responsive to innocuous mechanical stimuli and had conduction velocities consistent with Aδ and Aβ fibre neurons.

The afferent populations activated by icilin are consistent with populations of cold-sensitive neurons, including both low-threshold cold-sensitive afferents, which in rodents are predominantly C fibre neurons (Fang et al., 2005; Iggo, 1969; Iriuchijima & Zotterman, 1960; Lynn & Carpenter, 1982; Leem et al., 1993), and cold-sensitive nociceptive neurons, which in rodents are associated with Aδ and C fibres (Fang et al., 2005; Koltzenburg et al., 1997; Lynn & Carpenter, 1982; Leem et al., 1993). The lack of effect of icilin on Aβ fibres shows that the effect of icilin on afferents is indeed specific to a subset of neurons. These recordings are also consistent with icilin-mediated activation of TRPM8-expressing populations. TRPM8 appears to be expressed selectively in small-diameter sensory neurons, consistent with expression in C and possibly Aδ fibre neurons (Kobayashi et al., 2005; Okazawa et al., 2004; Peier et al., 2002b; results section 4.5).

Similar increases in firing frequency in C fibre neurons were observed following topical application of menthol to the receptive field area. This was performed as part of the study investigating the effect of treatment with intrathecal antisense or mis-sense oligonucleotides directed at TRPM8. In animals that had been treated with mis-sense against TRPM8, and therefore would be expected to still express TRPM8, menthol elicited increases in firing frequency in C fibre neurons. This indicates that icilin and menthol produce similar effects at the level of primary afferent neurons, supporting an effect on afferent firing mediated via their common target, TRPM8.

Similar effects of both menthol and icilin were observed in afferent recordings. These two agonists have marked differences in their mode of activation of TRPM8 channels as analysed in vitro. When applied to transfected cells, icilin activates TRPM8 with a delay of variable latency (~10 s) (Andersson et al., 2004; Chuang et al., 2004) and requires an increase in intracellular Ca^{2+} for full agonist action (Chuang et al., 2004; McKemy et al., 2002), whereas menthol shows neither of these properties. At the molecular level, different parts of the TRPM8 receptor are critical for conferring menthol and icilin sensitivity: the putative TM segment 2 and part of

the C-terminal domain confer menthol sensitivity (Bandell et al., 2006); whereas the intracellular loop between TM segments 2 and 3 is critical for icilin-activation of the channel (Chuang et al., 2004), indicating distinctive mechanisms of activation.

Despite these differences in the molecular mechanism of activation, menthol and icilin showed similar effects in in vivo recordings of saphenous nerve fine afferents. This could be partly explained by experimental differences – for example, the latency associated with icilin activation of TRPM8 recorded in whole-cell voltage-clamp recordings of TRPM8-expressing cells could not be measured in these experiments, as a far more substantial delay would occur due to the time required for topically applied icilin (or menthol) to reach its target receptors and activate afferents. Nevertheless, the remarkably similar effects of these agonists on afferent recordings suggest that the differences between these two agonists at the levels of the single channel may not significantly affect the responses of afferent neurons to these drugs in vivo.

5.5 Analgesia produced by central activation of TRPM8

Intrathecal application of icilin and menthol also reversed ipsilateral behavioural sensitisation in the CCI model, for up to 55 minutes following injection. The ED_{50} of icilin was 0.17 ± 0.02 and 0.31 ± 0.02 nmol, for reversal of thermal and mechanical sensitisation respectively, again demonstrating the effectiveness of low doses of icilin. Intrathecal (-)-menthol exerted similar effects, supporting a TRPM8-mediated action. Therefore central activation of TRPM8 is also effective in reducing behavioural sensitisation, which suggests that the analgesia produced by TRPM8 activation may be centrally mediated, as it can be produced by central activation of TRPM8 receptors, downstream of the sensitised area. In agreement with topical data, intrathecal icilin had no effect in naïve animals or on contralateral thresholds, again demonstrating a selective effect on sensitised responses.

Intrathecal administration of icilin also reversed behavioural sensitisation in two other models of chronic pain studied.

Icilin effectively reversed the ipsilateral sensitisation that occurred following CFA injection into the hindpaw, thereby demonstrating that icilin can produce analgesia in

inflammatory pain states. Therefore, this mechanism is not confined to neuropathic states but represents a general mechanism of analgesia in sensitised pain states.

As discussed above (section 5.1), the lysolecithin model is a model of behavioural sensitisation induced by peripheral demyelination (Wallace et al., 2003). Peripheral demyelinating diseases are frequently associated with pain: chronic pain has been reported as a complication in 71% of patients sampled who suffered from forms of Charcot-Marie-Tooth syndrome (Carter et al., 1998), and in 54 – 84% of cases of Guillain-Barré syndrome (Asbury, 1990; Moulin et al., 1997). The effectiveness of icilin in the lysolecithin model suggests that TRPM8 activators could be a potential therapy for the pain associated with peripheral demyelinating diseases.

In additional experiments, intrathecal icilin reversed the withdrawal reflexes to 4°C cold induced by the lysolecithin model of demyelination-induced pain. The phenomenon of cold allodynia, in which normally innocuous cool temperatures produce a sensation of burning pain, is frequently observed in neuropathic pain conditions (Woolf & Mannion, 1999), and in peripheral demyelinating diseases (Ochoa & Yarnitsky, 1994). The attenuation of cold allodynia by the TRPM8 activator icilin strongly argues against a role for TRPM8 in nerve-injury induced cold allodynia, which is in agreement with data from this study and others (Katsura et al., 2006) showing that TRPM8 knockdown has no effect on cold allodynia. This result suggests that TRPM8 activators may be effective analgesics in neuropathic pain conditions in which cold allodynia is observed. These experiments were performed as an initial study of the effects of icilin on cold allodynia. In the future it would be beneficial to examine the effects of icilin on cold allodynia in additional models of sensitised pain states.

5.5.1 Comparison of the effects of intrathecal and systemic icilin

When icilin is administered intraperitoneally in rodents, vigorous shaking known as "wet-dog shakes" is elicited (Wei & Seid, 1983; Werkheiser et al., 2006a,b). I did not observe this phenomenon following intrathecal injection. This could be due to the fact that the maximum intrathecal dose was 10 nmol, whereas wet-dog shakes were reported following doses of ~500 nmol. It could also be due to the fact that the drug

is confined to the spinal cord following intrathecal administration, rather than becoming systemic (Zhang et al., 2001).

The "wet-dog shakes" phenomenon resembles the effects of morphine withdrawal syndrome in rats (Collier et al., 1974), and it was reported to be antagonised following subcutaneous pre-treatment with agonists of μ- or κ-opioid receptors, but only with agonists that are capable of crossing the blood-brain barrier, suggesting a central effect (Werkheiser et al., 2006a). Microdialysis experiments showed that intraperitoneal administration of icilin increased glutamate levels in the dorsal striatum (Werkheiser et al., 2006b). Although the mechanism is unclear, these results suggest that the shaking phenomenon elicited by icilin is mediated in the brain, and can be modulated by opioidergic transmission. Another study found that intracerebroventricular administration of adenosine and 2-chloroadenosine produced dose-dependent inhibition of icilin-induced shaking (Tse & Wei, 1986). The relationship between the actions of adenosine and of opioids on the action of icilin is unclear, but both of these studies suggest that the shaking behaviour elicited by icilin is produced centrally, in the brain, and therefore may be distinct from the action of icilin at TRPM8 in peripheral sensory neurons.

5.6 TRPM8 as a specific mediator of the actions of icilin and menthol

Neither icilin nor menthol is a completely specific activator of TRPM8. In addition to its identified agonist properties at TRPM8, menthol is also an agonist of the TRPV3 warm receptor and an antagonist of TRPA1 (Macpherson et al., 2006). However, Ca^{2+}-imaging data show that bath application of menthol can effectively evoke responses in TRPV3-expressing cells only at concentrations reaching ~ 1 – 2 mM, whereas it acts on TRPM8 with an EC_{50} of 30 μM in similar conditions (Macpherson et al., 2006), and therefore this effect is unlikely to be important in the low doses of menthol used in the experiments in this thesis. Similar experiments, however, show an IC_{50} value of 68 μM for menthol-mediated inhibition of TRPA1 (following TRPA1 activation by 75 μM cinnamaldehyde) (Macpherson et al., 2006). This value is in a similar range to the EC_{50} of menthol acting at TRPM8, and is therefore likely to be a factor in need of consideration in the overall effect of low-

dose menthol on sensory neurons (Macpherson et al., 2006). It should be noted that the doses quoted here in experiments using bath application on transfected cells are naturally lower than those required to exert effects following topical or intrathecal application in whole animal studies.

Menthol has also been shown to induce Ca^{2+} release from intracellular stores, an effect which has been demonstrated in prostate cancer cell lines (Thebault et al., 2005; Zhang & Barritt, 2004), skeletal muscle (Palade, 1987), and sensory neurons (Tsuzuki et al., 2004). This effect was initially thought to be due to menthol acting at TRPM8 located on intracellular membranes, which has been demonstrated immunohistochemically in some experiments (Thebault et al., 2005). However, recent evidence suggests that this menthol-induced Ca^{2+} release pathway is TRPM8-independent, as menthol-induced Ca^{2+} release was observed in non-TRPM8-expressing HEK293, CHO and COS cells (Mahieu et al., 2006). Furthermore, this release was inhibited by cooling and potentiated by warming, the opposite of what would be expected by a TRPM8-mechanism, and was mimicked by compounds structurally similar to menthol such as linalool, but not by the stronger TRPM8 agonist icilin (Mahieu et al., 2006), suggesting that menthol exerts effects on intracellular Ca^{2+} stores via a TRPM8-independent mechanism. This effect was observed with menthol doses between 1 – 3 mM (bath application to whole cells), which is significantly higher than the concentration required to activate TRPM8 in similar conditions, suggesting that this action of menthol can be avoided, and TRPM8 selectively activated, by the use of low doses of menthol.

Icilin is also an agonist at receptors other than TRPM8. Icilin is an activator of TRPA1, but acts with lower potency at this receptor compared with TRPM8, requiring concentrations of 25 μM for activation in transfected oocytes, whereas TRPM8 transfected cells were activated by 1 μM icilin (Story et al., 2003), suggesting that low doses of icilin will selectively activate TRPM8. However, icilin appears to act as a partial agonist of TRPA1 (S. Bevan, personal communication) and it is possible that therefore it could function as a competitive antagonist of TRPA1 agonists.

Icilin is also reportedly an agonist acting at the ENaCδ channel subunit (epithelial sodium channel δ) (Yamamura et al., 2005). However, this effect is unlikely to

impact on the experiments of this thesis: firstly because icilin has a much lower potency acting at this channel, with an EC_{50} of 30 μM compared with 0.2 μM at TRPM8 in similar conditions; and secondly because the ENaCδ subunit is principally expressed in the brain, with relatively little expression in sensory neurons (Yamamura et al., 2005).

The interactions of icilin and menthol with these alternative targets or with other currently unidentified targets, appear not to play a role in icilin and menthol-induced analgesia. Firstly, both icilin and menthol elicited analgesia, suggesting that these drugs are working through their common target, TRPM8, rather than their individual alternative targets. Secondly, the effects of these drugs were eliminated by specific knockdown of TRPM8. Pretreatment with an antisense oligonucleotide to knockdown expression of TRPM8 abolished the analgesic effect of topical icilin in CCI animals, whereas mis-sense treatment was ineffective. This result suggests that the analgesia mediated by icilin and menthol is indeed mediated through the TRPM8 receptor. Following TRPM8 knockdown, there was no effect of icilin on behavioural thresholds or on observed behaviours in general, suggesting that icilin does not exert significant effects on behavioural thresholds through non-TRPM8 mechanisms. Therefore, the evidence is consistent with a local analgesic mechanism at least partially dependent on TRPM8, and which can be recruited by agonists such as icilin and menthol in the correct doses.

Antisense knockdown of TRPM8 abolished activation of the subset of slowly-conducting afferents by topical icilin and menthol. This result strongly supports the idea that the activation of afferents by topical icilin and menthol is indeed mediated specifically through activation of TRPM8 expressed in afferent neuron terminals. The abolition of the effect of icilin and menthol on afferents also supports the effectiveness of antisense treatment, suggesting that functional TRPM8 in primary afferent peripheral terminals is indeed lost following intrathecal antisense treatment to knock down sensory neuron expression of TRPM8. Responses evoked by the TRPV1 agonist, resiniferatoxin, were unaltered, demonstrating the specificity of antisense treatment to TRPM8, rather than affecting all sensory TRP receptors, and rather than causing a generalised toxic effect on sensory neurons.

TRPM8 antisense treatment had no effect alone on baseline behavioural values or on CCI-induced thermal hyperalgesia and mechanical and cold allodynia. Therefore TRPM8 does not appear to be involved in setting thresholds for normal or sensitised nociceptive responses. The lack of effect of TRPM8 antisense knockdown on CCI-induced cold allodynia suggests strongly that abnormal responses to noxious cold in nerve-injury models are mediated through non-TRPM8 mechanisms, in agreement with another study, which also showed that antisense knockdown of TRPM8 had no effect on the development of cold allodynia in the SNL model of neuropathic pain (Katsura et al., 2006).

5.7 TRPM8 expression and its alteration in neuropathic pain states

5.7.1 TRPM8 expression in DRG

In order to analyse the expression of TRPM8 in different subpopulations of DRG sensory neurons I examined TRPM8 co-localisation with NF-200, which selectively marks myelinated afferents (Lawson et al., 1984; Lawson & Waddell, 1991) and with peripherin, an intermediate filament protein which selectively labels unmyelinated afferents (Amaya et al., 2000). In naïve animals, TRPM8 expression was observed in a subpopulation (8.3 ± 0.2 %) of peripherin-positive DRG neurons, which all had small-diameter cell bodies (mean diameter of 18.4 ± 0.7 μm). TRPM8 was minimally expressed in myelinated fibres (1.3 ± 0.5 %), which all had small-diameter cell bodies (mean diameter: 19.7 ± 0.8 μm) and are therefore assumed to be Aδ fibres. These results are in agreement with previously published results, which show TRPM8 expression in a subpopulation of small-diameter primary afferents. Peier et al. found TRPM8 mRNA expression in 5 – 10% of DRG neurons, with a diameter consistent with that for C fibre neurons in the mouse (Peier et al., 2002b), and McKemy et al. also observed TRPM8 mRNA in under 20% of DRG neurons, predominantly small-diameter neurons (mean diameter of 18.2 μm) (McKemy et al., 2002). Another group, using both high-sensitivity cRNA hybridisation and immunohistochemistry, observed TRPM8 in approximately 23% of DRG neurons (Katsura et al., 2006; Kobayashi et al., 2005), and this group also observed TRPM8 mRNA in up to 19% of NF-200-positive afferents in naïve animals (Kobayashi et al.,

2005). The reason for the somewhat higher expression levels observed by this group, compared with my results, is unclear. It is possible that the differences with the in situ hybridisation experiments could be explained if not all of the mRNA observed is expressed as protein. The immunohistochemistry studies utilised different antibodies, and may have used different threshold criteria for what constituted a positive cell, which could account for the different levels of expression observed by this group and by ourselves.

Overall however, the observation of TRPM8 expression predominantly in small-diameter, unmyelinated sensory neurons, is in broad agreement both with other expression studies, and with the afferent recording experiments, where it was observed that topical icilin and menthol selectively activated a population of mainly C fibre afferents, with some Aδ neurons.

As described in the introduction, changes in the expression of a variety of proteins occur in primary afferent neurons following peripheral nerve damage. I therefore analysed tissue from CCI animals to see whether TRPM8 levels are altered in this model. Both Western blot and immunohistochemistry showed a significant increase in TRPM8 protein levels in ipsilateral DRG following CCI.

In DRG neurons, I observed an increase in TRPM8 expression in both peripherin-positive neurons (an increase of 86% from naïve levels) and especially prominently in NF-200 positive cells, from a naïve level of 1.3 ± 0.5%, to 7.9 ± 1.2% ipsilateral to CCI. This upregulation in co-expression with NF-200 from a naïve level of almost zero suggests that TRPM8 expression in myelinated afferents is significantly altered in neuropathy, so that it becomes expressed in a population of cells, which do not normally express this receptor.

Somewhat different findings to ours were reported in the SNL model, where levels of TRPM8 mRNA and protein did not change in the spared L4 DRG, and levels of TRPM8 mRNA decreased in the L5 DRG, in tissue taken at the time of peak behavioural sensitisation (Katsura et al., 2006). However, Katsura et al. did not study co-expression of TRPM8 with markers of myelinated and unmyelinated cells, and therefore it is possible that the pattern of TRPM8 expression was altered in a similar manner to ours, without changes in the overall expression level. Alternatively,

differences between the SNL and CCI models could account for these different results.

The functional consequences of the significant alterations in TRPM8 expression that I observed in the CCI neuropathic pain model are not entirely clear. The increased expression of TRPM8 ipsilateral to CCI could at least partially account for the selective analgesic effect of icilin and menthol on ipsilateral responses. The alteration in expression pattern could also be important: it is possible that the analgesia produced by icilin is selectively mediated by the Aδ fibre population in which TRPM8 is newly expressed following CCI, and this would explain the selectivity of analgesia to the ipsilateral responses. However, icilin also reversed sensitisation induced by intrathecal injection of NMDA or cinnamaldehyde. In these models, sensitisation develops within 15 minutes following intrathecal injection, which is too short a time period for alterations in protein expression in the DRG to occur. These results suggest that an increase in TRPM8 expression or a change in the pattern of TRPM8 expression is not required for an analgesic effect of TRPM8 activation. The functional consequences of the change in TRPM8 expression observed in the CCI model therefore remain unclear. However, the increased TRPM8 expression, while not critical for mediating TRPM8-induced analgesia, could nevertheless facilitate this phenomenon. The increase in TRPM8 levels and the resultant facilitation of analgesia could represent a coping mechanism for sustained long-term painful conditions.

In future experiments it would be interesting to examine TRPM8 expression in the CFA model by immunohistochemistry. Western blots of tissue from CFA-treated animals showed no changes between ipsilateral and contralateral, or between CFA-treated and saline-treated DRG tissue. However, it is possible that the pattern of TRPM8 expression alters in the CFA model, with an upregulation in Aδ fibres similar to that seen in the CCI model, but with no obvious change in the overall level, and therefore it would be interesting to examine this expression pattern with immunohistochemistry experiments. Similarly, although TRPM8 expression in the lysolecithin model of demyelination was not examined, future experiments could address possible changes in expression in this model. It would also be interesting to investigate the timecourse of changes in TRPM8 expression, as the results reported

above examined tissue taken from animals at the peak of neuropathic sensitisation, and stages of intermediate neuropathic development were not examined.

No experiments have as yet addressed the expression of TRPM8 in human neurons. It is possible that there could be differences in afferent populations expressing TRPM8 between humans and rodents, and these differences could affect the ability of TRPM8 agonists to induce analgesia. It is known that there are significant differences between rodents and humans with regard to populations of cold-sensitive afferent neurons. Innocuous cold-sensitive afferents are reported to be mainly or exclusively C fibres in rodents (Iggo, 1969; Iriuchijima & Zotterman, 1960; Lynn & Carpenter, 1982; Leem et al., 1993), but in primates, innocuous cold-sensitive afferents are exclusively Aδ fibres in glabrous skin and a mixture of Aδ and C fibres in hairy skin (Campero et al., 1996; Darian-Smith et al., 1973; Iggo, 1969). TRPM8-expressing populations in humans would therefore be expected to comprise a much greater proportion of A fibres than in rodents, and this may affect the mechanism of TRPM8-mediated analgesia.

The present study only addressed co-expression with the markers NF-200 and peripherin. Future studies could address co-expression of TRPM8 with other markers of afferent neurons, in order to identify in more detail the neuronal populations that express TRPM8. Co-expression studies to date have indicated that TRPM8 is not co-localised with IB-4 or CGRP (Peier et al., 2002b), but that 98% of TRPM8-expressing neurons express the NGF receptor trkA (Kobayashi et al., 2005, Peier et al., 2002b). There is still controversy regarding the extent of possible TRPM8 co-localisation with TRPV1. In situ hybridisation and immunohistochemical studies have reported that between 0% (Kobayashi et al., 2005; Peier et al., 2002b) and 29% (Okazawa et al., 2004) of TRPM8-expressing cells also express TRPV1, and studies of cultured DRG neurons have similarly reported a varying degree (up to 50%) of overlap in capsaicin and menthol responsiveness (Babes et al., 2004; McKemy et al., 2002; Xing et al., 2006). Therefore, this issue is still not fully resolved. In addition, the degree of TRPM8 and TRPV1 co-localisation may alter in chronic pain states, and this has not been studied at all. Increased levels of NGF have been reported to correlate with increased TRPV1/TRPM8 co-expression (Babes et al., 2004; Story et al., 2003) and therefore, given the altered exposure to neurotrophic factors in

neuropathic pain states (Pezet & McMahon, 2006) it is plausible that alterations in co-expression could occur in such pain states. Future experiments could address these issues.

5.7.2 TRPM8 expression in the spinal cord

Electrophysiological studies of primary afferent-dorsal horn neuron synapses in spinal cord slices (Baccei et al., 2003) and in DRG-dorsal horn neuron co-cultures (Premkumar et al., 2005; Tsuzuki et al., 2004) have demonstrated a facilitatory effect of the TRPM8 agonist menthol at this synapse. Menthol elicited an increase in the frequency, although not the amplitude, of mEPSCs at the primary afferent-dorsal horn neuron synapse, which is suggestive of a presynaptic mechanism to cause increased glutamate release from the afferent neurons. The effect of menthol on dorsal horn synapses suggested that TRPM8 protein is also present in the spinal cord, on the terminals of primary afferents.

Analysis with both Western blot and immunohistochemistry revealed that TRPM8 protein is found in the spinal cord. TRPM8-immunoreactivity was observed in the superficial laminae of the spinal cord, and was almost entirely abolished by dorsal rhizotomy, indicating a localisation largely on the terminals of primary afferents. Therefore the effects of TRPM8 agonists at the primary afferent-dorsal horn neuron synapse, and in intrathecal drug tests, are presumably mediated by TRPM8 receptors located on the presynaptic terminals of afferents. The distribution of TRPM8-expressing afferent terminals in superficial laminae is consistent with TRPM8 expression by C and Aδ fibre neurons, which terminate predominantly in superficial laminae (Braz et al., 2005; Craig, 2003; Willis & Coggeshall, 1991). The pattern of TRPM8-immunoreactivity I observed in spinal cord is similar to that reported for the heat-sensitive TRPV1 receptor, which is similarly localised in superficial laminae and eradicated following dorsal rhizotomy (Tominaga et al., 1998), suggesting that sensory TRP receptors may be commonly expressed on the central terminals, as well as the peripheral terminals, of afferent neurons.

The increased level of TRPM8 seen in DRG tissue ipsilateral to CCI was also observed in immunohistochemical experiments in the spinal cord and in Western blots of a spinal cord preparation with an enriched membrane component. Therefore

the increase in TRPM8 in sensory neuron cell bodies does appear to correlate with increased expression at the central terminals of primary neurons in the spinal cord. This increased expression could facilitate the effect of TRPM8 agonists administered intrathecally in CCI animals.

5.8 TRPA1 activation produces hyperalgesia

In contrast to the analgesic role of TRPM8, I found that topical application of the selective TRPA1 activator cinnamaldehyde, or intrathecal application of the TRPA1 agonists cinnamaldehyde, allicin and diallyl disulphide induced thermal and mechanical hyperalgesia in naïve animals. Intrathecal cinnamaldehyde also sensitised contralateral responses and increased ipsilateral sensitisation in the CCI model. This effect of cinnamaldehyde was blocked by co-injection of the broad-spectrum TRP channel blocker, ruthenium red, which blocks TRPA1 (Macpherson et al., 2005) but not TRPM8 (Reid, 2005; Weil et al., 2005), in support of a TRPA1-mediated effect of cinnamaldehyde. Co-injection of ruthenium red with icilin had no effect on the analgesic effect of icilin, again supporting the idea that icilin does not exert its analgesic effect through the TRPA1 channel.

These results and those of other groups define a role for TRPA1 in transduction of noxious chemical stimuli. TRPA1 agonists such as allicin and diallyl disulphide (the active ingredients in garlic), allyl isothiocyanate (the active ingredient in mustard and horseradish), and acrolein (a toxic chemical present in tear gas, vehicle exhaust, and smoke) are well known for their production of a painful, pungent sensation when applied topically or orally (Bandell et al., 2004; Bautista et al., 2005, 2006; Jordt et al., 2004; Macpherson et al., 2005). TRPA1 is also activated indirectly by the endogenous proalgesic chemical bradykinin, which acts on BK_2 receptors to cause activation of TRPA1, through an imprecisely-defined pathway that is dependent on PLC activity (Bandell et al., 2004). Cinnamaldehyde (10%, equivalent to 75 mM) applied topically to the forearm in human subjects induces spontaneous pain, and thermal and mechanical hyperalgesia (Namer et al., 2005); intraplantar cinnamaldehyde (doses of 6.6 and 21.7 μg, delivered in 10 μl volume) causes licking and flinching behaviour in mice, and the development of thermal hyperalgesia

(Bandell et al., 2004). Studies of TRPA1 knockout mice showed decreases in the licking and flinching behaviour evoked by topical mustard oil (Bautista et al., 2006), intraplantar mustard oil (Kwan et al., 2006) or intraplantar bradykinin (Bautista et al., 2006; Kwan et al., 2006). These results and ours point to a critical role for the TRPA1 receptor in mediating the response to multiple noxious chemical stimuli, and in evoking nociceptive responses.

In humans, the sensory effect produced by chemical activators of TRPA1 generally includes a burning hot feeling (in contrast to the purported role of TRPA1 in mediating cold sensation). So intuitively, TRPA1 agonists might be expected to produce a thermal hyperalgesia. However, the reason for the mechanical hypersensitivity observed with cinnamaldehyde is less clear. It is possible that TRPA1 is expressed in polymodal nociceptors, and that activation of TRPA1 produces a sensitisation of the nociceptor terminal, allowing activation by lower levels of all activating stimuli, including thermal and mechanical stimuli. There could also be an induction of central sensitisation, following stimulation of TRPA1-expressing nociceptors, facilitating both thermal and mechanical inputs in the spinal cord. Alternatively, TRPA1 has been proposed to be involved in mechanotransduction (Introduction section 1.25.1), and therefore it is possible that a facilitation of TRPA1 produced by the agonist cinnamaldehyde could facilitate mechanotransduction by this receptor, contributing to mechanical hypersensitivity.

The behavioural sensitisation produced by administration of TRPA1 activators was blocked by icilin, suggesting that TRPM8 activation directly opposes the effect of TRPA1 activation. This result also demonstrates that TRPM8 activation produces analgesia in a sensitised pain state with a comparatively short timecourse. The models investigated above – CCI, CFA and lysolecithin – all develop over a period of days, whereas cinnamaldehyde-induced sensitisation is apparent within 15 minutes following intrathecal injection. The action of icilin on cinnamaldehyde-induced sensitisation indicates that although the effect of icilin is selective to a sensitised pain state, it does not require chronic changes in nervous system function (such as alterations in gene and protein expression, which would require longer than 15 minutes to occur).

It is widely agreed that TRPM8 and TRPA1 are not co-expressed (Kobayashi et al., 2005; Nagata et al., 2005; Story et al., 2003), and so the opposing effects of TRPM8 and TRPA1 agonists must be assumed to be mediated by different neurons. This implies that, in order for TRPM8-activators to attenuate TRPA1-induced hyperalgesia, there must be a centrally-mediated mechanism of analgesia.

Recent results show that cinnamaldehyde, in addition to activating TRPA1, also acts as an antagonist of the TRPM8 receptor (Macpherson et al., 2006). However, much higher concentrations are required for the antagonist action of cinnamaldehyde at TRPM8 in comparison with the concentrations required to activate TRPA1: antagonistic effects of cinnamaldehyde on TRPM8 were only achieved at a bath concentration of 1.5 mM in whole cell recordings (Macpherson et al., 2006), and therefore it is unlikely that the 1.5 mM concentration applied topically to the skin here and intrathecally will have exerted much effect on TRPM8. Icilin itself interacts with the TRPA1 channel, but, as discussed above, with a significantly lower potency than at TRPM8 (Story et al., 2003). Furthermore, the analgesic effect of icilin shown in behavioural experiments is entirely different from the pro-nociceptive profile of TRPA1 activators, and icilin had no effect on behavioural responses in animals which had undergone antisense-knockdown of TRPM8, suggesting that, at the doses used, icilin had no effect on behavioural responses through TRPA1-mediated mechanisms

5.9 Central mediation of TRPM8-induced analgesia through Group II/III mGluRs

Central activation of TRPM8 was analgesic, suggesting that central processing mechanisms may be involved in the analgesic effect of TRPM8 activation. The idea of central processing is also indicated by the fact that TRPM8 is expressed by a relatively small population of innocuous cold-sensitive neurons and possibly by some cold-sensitive nociceptors. Therefore it is unlikely that TRPM8 is expressed by a large proportion of the fibres that mediate the sensitised responses to thermal and mechanical stimuli, suggesting that, in order for TRPM8 activation to produce a

strong reversal of thermal and mechanical sensitisation, a central mechanism must be involved.

TRPM8 analgesia appears to be independent of spinal opioidergic mechanisms, as it was not blocked by spinal administration of the non-selective opioid antagonist naloxone. This is perhaps unsurprising, as icilin was highly effective in producing analgesia in a neuropathic pain model, and the effects of opioids on responses to painful stimuli and on the responses of dorsal horn neurons are decreased in neuropathic pain states (Dickenson & Suzuki, 2005; Moulin, 2006; Woolf & Mannion, 1999).

Instead, the analgesia induced by intrathecal icilin and menthol was found to be dependent on Group II/III metabotropic glutamate receptors, as it was reversed by co-injection of antagonists of these receptors. The mGluR action is not due to a non-specific interaction between the two drugs, and also appears to be engaged downstream of TRPM8 activation, as intrathecal injection of Group II/III mGluR antagonists reversed the analgesic effect of peripheral menthol and icilin application, and of skin cooling to 16°C. The antagonists were without effect alone in either CCI animals or naïve animals, suggesting that Group II/III mGluRs are not active tonically but are specifically activated downstream of TRPM8 activation. This evidence, together with supporting electrophysiological evidence, strongly suggests a role for Group II/III mGluRs in the mediation of TRPM8-induced analgesia.

Results further showed that agonists of Group II/III mGluRs mimicked the effects of icilin, selectively inhibiting the sensitised responses ipsilateral to CCI. Previous experiments have shown that intrathecally administered Group II mGluR agonists reverse sensitisation in models of inflammatory (Dolan & Nolan, 2002; Fisher et al., 2002; Simmons et al., 2002) and neuropathic pain (Fisher et al., 2002; Simmons et al., 2002), and that Group II mGluR agonists reverse capsaicin- or inflammation-induced sensitisation of spinal projection neuron responses to mechanical and electrical stimulation, with little effect on normal responses (Neugebauer et al., 2000; Stanfa & Dickenson, 1998). Therefore, these receptors are implicated in the selective reversal of sensitised pain states, and in agreement with this a selective reversal of sensitised ipsilateral responses in CCI animals was observed.

Somewhat differently to the Group II mGluRs, several studies suggest that Group III mGluRs inhibit both normal and sensitised responses to sensory stimuli. Agonists of these receptors inhibit normal responses to mechanical brush and pinch stimuli and electrical stimulation, as well as the sensitisation of these responses produced by peripheral capsaicin or inflammation (Gerber et al., 2000; Neugebauer et al., 2000; Stanfa & Dickenson, 1998). However, another study showed that agonists selectively inhibit sensitised behavioural responses in the SNL model of neuropathy, with no effect in normal rats, and inhibited responses of dorsal horn projection neurons to mechanical stimulation in SNL but not in naïve rats, suggesting that Group III mGluR inhibitory function may be elevated in neuropathy (Chen & Pan, 2005). This is consistent with my behavioural observations, showing a selective reversal of ipsilateral sensitisation in CCI animals, with no effect on contralateral thresholds.

Extracellular recordings of single dorsal horn neurons in CCI animals showed results that mirrored behavioural findings. Application of icilin to part of the neuronal receptive field inhibited the sensitised responses of ipsilateral neurons to motorised rotating brush, with no effect on contralateral neurons. It is reasonable to assume that this brush stimulus represents a noxious effect ipsilateral to CCI. In neuropathic pain syndromes, allodynia develops so that innocuous mechanical stimuli such as brushing of the skin are perceived as noxious (Koltzenburg et al., 1994; Maihofner et al., 2003), and in peripheral nerve injury models, the responses of spinal cord neurons to innocuous brush stimuli are sensitised, and show a significantly higher discharge frequency ipsilateral to injury (Leem et al., 1995; Palecek et al., 1992), indicating that the response to innocuous mechanical input has become sensitised and that this innocuous brushing now represents a noxious stimulus.

The neurons recorded from were multireceptive neurons, identified by their response to both innocuous brush and a noxious pinch stimulus, and a majority were located in LIII/IV, with some (25%) in LI. The effect of icilin on LIII/IV neurons indicates that mechanisms must be engaged downstream of TRPM8 activation, as immunohistochemistry showed that TRPM8 expression is confined to the superficial laminae of the dorsal horn. The Group II/III mGluRs may be this downstream mechanism, as evidenced by behavioural experiments and by the fact that the Group

III mGluR selective antagonist UBP 1112 blocked the inhibitory effect of topically applied icilin on ipsilateral neuron responses. UBP 1112 was used as an example of a Group II/III mGluR antagonist, and the effects of Group II selective antagonists were not examined. Further experiments could address this issue, to see whether these antagonists produced a similar effect to UBP 1112.

The results obtained suggest that the Group II/III mGluRs play an important role in mediating TRPM8-induced analgesia. This does not appear to be due to a change in expression however, as no change in expression levels of any of these - mGluR2/3, mGluR4 and mGluR7 - was observed in Western blots of DRG and spinal cord in CCI and naïve animals, or in immunohistochemical analysis of mGluR7 in DRG.

5.10 Possible postsynaptic component of TRPM8-mediated analgesia

To further investigate the effect of TRPM8 activation on sensitised responses in the spinal cord, the effect of icilin was examined on sensitisation evoked by intrathecal NMDA application. The NMDAR is critically implicated in the generation of increased spinal cord excitability and sensitised pain states, as demonstrated by results showing that the facilitation, although not the response itself, of dorsal horn neurons following repeated stimulation of nociceptive fibres, is selectively reduced by NMDAR antagonists (Davies & Lodge, 1987; Dickenson & Sullivan, 1987; Dickenson & Aydar, 1991; Dougherty & Willis, 1991; Woolf & Thompson, 1991), and that intrathecally administered NMDAR antagonists attenuate neuropathic pain behaviours (Chaplan et al., 1997; Mao et al., 1992; 1993; Tal & Bennett, 1993). Intrathecal administration of NMDA produces hyperalgesia in naïve animals (Aanonsen & Wilcox, 1987; Kolhekar et al., 1994; Liu et al., 1997) and therefore this was used as a model of a spinally-generated, sensitised pain state, in which to investigate the effect of icilin.

Co-injection of icilin with NMDA prevented this development of sensitisation. In CCI animals, co-injection of icilin also prevented the further increase in ipsilateral sensitivity evoked by lower doses of NMDA that were insufficient to affect

contralateral or naïve responses. Therefore TRPM8 activation is also effective in producing a reversal of NMDA-mediated sensitisation.

The majority of NMDARs are located postsynaptically, and are implicated in postsynaptic facilitation of spinal cord neurons (Dickenson & Sullivan, 1987). Therefore, the fact that icilin reversed NMDA-induced sensitisation suggests that icilin may act through a partially postsynaptic mechanism of action. However, NMDARs may also be located presynaptically on afferent terminals (Liu et al., 1994) and therefore it is possible that part of the action of intrathecal NMDA is mediated through these presynaptic receptors. It is thought that the activation of presynaptic NMDARs may contribute to the release of the neuropeptide substance P (SP) from a subset of primary afferents in the rat dorsal horn. Intrathecal NMDA induces internalisation of the SP receptor into dorsal horn neurons, which is an effect generally taken to indicate SP release (Mantyh et al., 1995), and this internalisation, and the hyperalgesic effect of intrathecal NMDA, are reduced by co-injection of SP receptor antagonists or by elimination of TRPV1-containing nociceptive primary afferents with capsaicin (Liu et al., 1997). These results therefore suggest that part of the nociceptive effect of NMDA is mediated by inducing SP release from primary afferents (Liu et al., 1997). However, it has also been suggested that presynaptic NMDARs can play an inhibitory role (Bardoni et al., 2004), as in spinal cord slice experiments, NMDA evoked primary afferent depolarisation (an electrophysiological phenomenon which results in primary afferent inhibition) and a depression of the frequency of action potential-evoked EPSCs and an increase in latency and in transmission failures (Bardoni et al., 2004), which are taken to indicate a presynaptic effect (Engelman & Macdermott, 2004). While activation of presynaptic NMDARs could contribute to the facilitatory effect of intrathecal NMDA, by stimulating SP release, or, conversely, could inhibit sensitisation by inhibiting glutamate release from nociceptive afferents, it is reasonable to assume that the effect of intrathecally-injected NMDA is mediated substantially via postsynaptic receptors, due to the high levels of postsynaptic receptors (Petralia et al., 1994; Tolle et al., 1993). Therefore, the complete block of NMDA-mediated sensitisation by icilin suggests that some of icilin's action may be mediated through a postsynaptic mechanism.

5.11 Schematic model of results

Figure 5.1 shows a schematic outline of a model which summarises the findings of this thesis. In this model, activation of TRPM8 by peripheral or central means causes the release of glutamate from TRPM8-expressing afferents, which then acts on Group II/III mGluRs, located presynaptically on nociceptive afferents and/or postsynaptically on spinal neurons, to result in attenuation of both neuronal sensitisation and pain behaviours. In support of this model, TRPM8-activating compounds have been shown to activate a subpopulation of afferents. Other experiments have shown that menthol increases mEPSC frequency, but not amplitude, at synapses between afferent and dorsal horn neurons in slices (Baccei et al., 2003) and in DRG-dorsal horn neuron co-cultures (Premkumar et al., 2005; Tsuzuki et al., 2004) which is presumably due to a activation of TRPM8-containing afferents and increased release of glutamate from presynaptic terminals. Peripheral or central administration of TRPM8 agonists produced analgesia and inhibition of dorsal horn neurons in sensitised pain states, and this was dependent on spinally located Group II/III metabotropic glutamate receptors.

A large proportion of Group II/III mGluR expression is indicated to be presynaptic on primary afferent terminals (Azkue et al., 2001; Carlton et al., 2001; Ohishi et al., 1995a,b), suggesting a presynaptic mechanism of action. However, as discussed above, inhibition of NMDA-induced sensitisation suggests an at least partially postsynaptically mediated mechanism.

This model represents a gating mechanism, in which signals from nociceptive afferents are inhibited by activity in other afferents, presumably cold-sensitive TRPM8-expressing afferents, via a central spinal mechanism. The ability of non-nociceptive information to gate nociceptive signalling via a spinal mechanism was first suggested in the gate control theory of Melzack & Wall (Melzack & Wall, 1965). In the mechanism identified here, cold-sensitive afferents appear to gate the activity of nociceptive afferents, via the activation of inhibitory mGluRs. Further questions regarding this mechanism, including its relationship to the gate control theory, and the role of pre and postsynaptic mGluRs, will be discussed further below in section 5.15.2

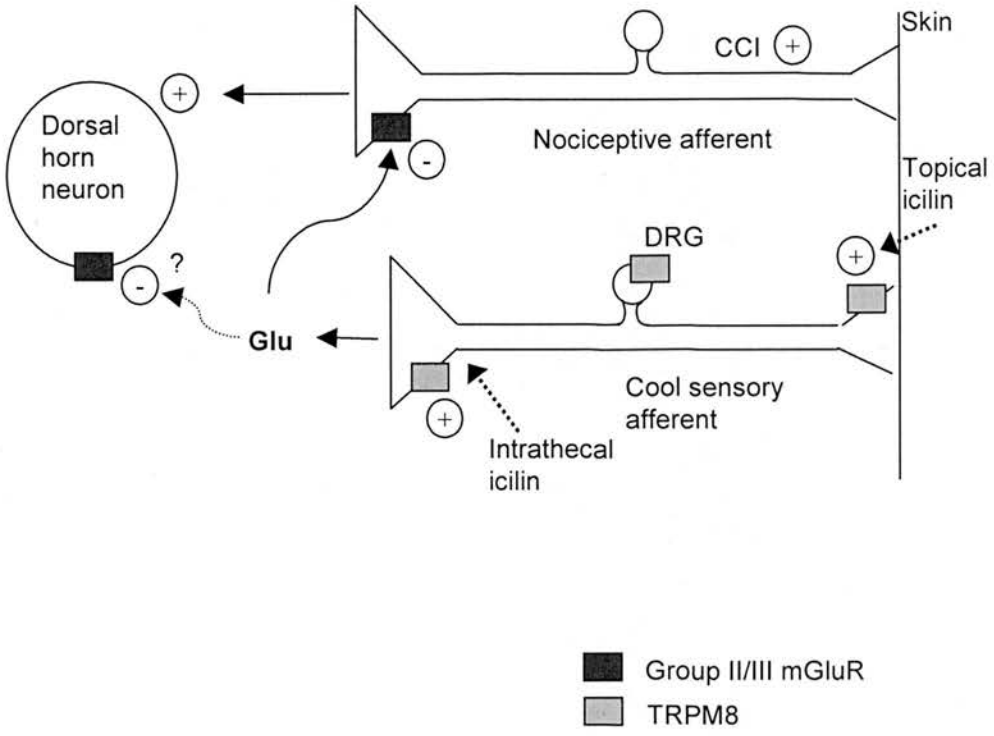


Figure 5.1 Schematic representation of a possible mechanistic basis for TRPM8-mediated-analgesia
In this simplified hypothetical model, activation of TRPM8 in a subpopulation of afferents, by icilin, menthol or moderate cooling leads to central synaptic release of glutamate (Glu) which then acts through inhibitory Group II/III mGluR receptors located either presynaptically on injury-activated nociceptive afferents or perhaps also postsynaptically on dorsal horn neurons, thereby attenuating neuropathic sensitisation

5.12 Overall significance of results

These results considered as a whole describe an analgesia which can be produced by activating TRPM8 receptors located on primary afferents, is mediated centrally through Group II/III metabotropic glutamate receptors in the spinal cord, and is specific to sensitised pain states. The key significance of these results is that they identify a mechanism underlying the effect of analgesia produced by cutaneous cooling and by menthol or mint oil, and identify a novel endogenous analgesic system which may be exploited for new clinical treatments of chronic pain. This study is also notable in that it is one of the first in vivo studies of the effects of TRPM8 activation. Extensive in vitro studies have characterised the properties of the channel (McKemy, 2005; Reid, 2005), and studies have also addressed the localisation of this receptor (Kobayashi et al., 2005; Okazawa et al., 2004; Peier et al., 2002b). However, this is the first time that the effects of TRPM8 activation have been examined in detail in vivo in afferent and spinal dorsal horn neuron recordings, and behavioural experiments.

The relationship of these findings to previous knowledge about the analgesia produced by cooling and mint oil, and their relevance to clinical applications, will be considered below.

5.13 Identification of a mechanism of analgesia produced by cutaneous cooling

These results identify a mechanistic basis for the phenomenon of cooling-induced analgesia, which has been known since ancient times (Adams, 2006; Sprengell, 1735; Siegel, 1970). Activation of TRPM8 by cooling or chemical agonists produces a strong analgesia in sensitised pain states, which is abolished by TRPM8 knockdown, suggesting that both the analgesic effect of cutaneous cooling, and the analgesic effect of topical menthol or mint oil, are at least partially mediated through activation of the TRPM8 receptor.

Other mechanisms have been proposed to account for cooling-induced analgesia. Cooling-induced inhibition of peripheral nociceptive transduction processes has been proposed as a mechanism (Koltzenburg et al., 1992), and it has been demonstrated

that cooling inhibits capsaicin-induced currents through TRPV1 in DRG cells (Babes et al., 2002), and inhibits capsaicin-induced stimulation of CGRP release in rat skin (Kichko & Reeh, 2004), although no studies have shown a specific inhibitory effect of cooling on receptors for non-thermal noxious stimuli. Cooling may also inhibit local inflammatory responses by promoting vasoconstriction. Very strong local cooling (<0°C), which produces a block of nervous conduction, inhibits pain perception by preventing conduction by nociceptive afferents (Abramson et al., 1966; Evans, 1981; Lee et al., 1978). The mechanism described in this thesis does not rule out other mechanisms such as these. In the analgesia produced by low (≤0°C) temperatures, mechanisms such as cold-induced block of nervous conduction, or the activation of descending inhibitory pathways elicited by noxious stimuli are likely to predominate. Similarly, in conditions in which peripheral tissue inflammation is an important factor, cooling-induced inhibition of TRPV1 and cooling-induced vasoconstriction may play a significant role.

However, by using selective activators of TRPM8, rather than physical cooling (and antisense knockdown of TRPM8), TRPM8 activation is strongly implicated in the analgesia produced by moderate cutaneous cooling. Furthermore, this effect (and that of moderate cutaneous cooling) is apparent in the CCI model of neuropathic pain, in which peripheral inflammation will play a relatively small role, as the injury is set up proximal to the periphery, and as neuropathic models are thought to rely predominantly on changes in afferent neurons and in the spinal cord, rather than on peripheral inflammation (Bridges et al., 2001; Ji et al., 2003; Campbell & Meyer, 2006).

The results described identify a powerful endogenous mechanism of analgesia that can be elicited by local stimulation of TRPM8 in sensitised pain states. This mechanism is active in neuropathic, inflammatory, demyelination-induced and chemical models of sensitised pain states, suggesting a general mechanism for coping in sensitised pain states. The extent to which this mechanism is activated naturally in sensitised pain states is unclear. Peripherally located TRPM8 can be activated by cool temperatures, and humans and animals could use local peripheral cooling as a means of relieving pain. However, as described in the introduction, chemical modulators of TRPM8 exist, and the central location of TRPM8 (in the

spinal cord) suggests that chemical agonists of TRPM8 must exist. In sensitised pain states, it is possible that endogenous chemical agonists could be released, which activate or facilitate TRPM8, partially inhibiting the increased pain signalling and allowing the animal to cope. This mechanism could have evolved as a coping mechanism, allowing the induction of local analgesia to permit animals to survive and deal with injury.

It is generally accepted that other mechanisms, at present poorly identified, also contribute to the sensory detection of cold temperatures (Madrid et al., 2006; Munns et al., 2006; Reid, 2005; Story et al., 2003; Viana et al., 2002, 2006). In addition to TRPA1, the role of which in cold sensitivity is still under debate, studies suggest additional cold-sensitive mechanisms. Populations of sensory neurons have been identified that respond to decreases in temperature within the range of 30 – 20°C, but not to activators of either TRPM8 or TRPA1 (Babes et al., 2004), and that do not express TRPM8 or TRPA1 (Babes et al., 2006). Additional cold-sensitive currents in sensory neurons have been identified, such as a hyperpolarising K^+ current which is deactivated by decreasing temperature (Reid & Flonta, 2001b; Viana et al., 2002). Additional ion channels have been shown to respond to decreases in temperature, including the K^+ channels TREK-1 and TREK-2 (Lesage et al., 2000; Maingret et al., 2000), and the epithelial Na^+ channel ENaC (Askwith et al., 2001). The role of such alternative transduction mechanisms in cooling-induced analgesia is currently unclear. It is possible that these other cold-activated currents could play a role in the analgesic effect of cutaneous cooling, but they presumably do not play a role in the analgesic effect of TRPM8-specific agonists, particularly as icilin and menthol had no effect following TRPM8 knockdown. On balance, the strong analgesic effect of TRPM8 activators suggests that this receptor may play a dominant role in cooling-induced analgesia. This strong analgesic effect of specific TRPM8 activators, together with the difficulty associated with applying precisely defined cold temperatures and the fact that more intense cold temperatures can be noxious, suggests that chemical agonists of TRPM8 may be a far more specific and powerful means of producing clinical analgesia than physical cooling.

5.13.1 Argument against a desensitisation mechanism of icilin and menthol-induced analgesia

A possible explanation for analgesia mediated by menthol or icilin administration could be that these agonists cause desensitisation of the TRPM8 receptor. In this scenario, TRPM8 would be predominantly located on nociceptive neurons, contributing to nociceptive transmission and to sensitised pain behaviours, and agonists would cause TRPM8 desensitisation, resulting in inhibition of nociceptive signalling. However, this is unlikely to account for the effects observed here for the following reasons. Firstly, desensitisation would be a purely peripheral mechanism, occurring at the peripheral receptor, with no requirement for effects downstream, but the analgesic effect of icilin appears to be dependent on metabotropic glutamate receptors in the spinal cord. Secondly, antisense knockdown of TRPM8 had no effect on behavioural thresholds or on the sensitisation of these that occurs ipsilateral to CCI. If the TRPM8 receptor was involved in nociceptive signalling, then antisense knockdown itself would be expected to affect behavioural sensitivity to noxious stimuli. Thirdly, saphenous nerve recordings demonstrated that icilin and menthol caused a prolonged stimulation of afferent firing, that was evident over 12 minutes following application of the drug, arguing that icilin and menthol, at the doses used, do not have a rapid desensitising effect on the firing of afferents. Fourthly, agonist-mediated desensitisation of TRPM8 would only be expected to affect TRPM8-expressing neurons. As TRPM8 is expressed by a relatively small proportion of DRG neurons, and is not co-expressed with several markers of nociceptive neurons (Peier et al., 2002b), it is unlikely that TRPM8 will be expressed to a great extent in the neuronal populations that mediate the CCI-sensitised responses to thermal and mechanical stimuli. Therefore desensitisation of TRPM8 would not be likely to account for the strong reversal of thermal and mechanical sensitisation observed with TRPM8 agonists. On balance, it appears highly unlikely that icilin and menthol-mediated analgesia occurs through desensitisation of TRPM8.

5.13.2 Argument against a counter-irritation-induced mechanism of analgesia

A mechanism which has previously been proposed to account for the analgesic effect of topically applied menthol is that of counter-irritation. The principle of

counter-irritation is that pain can be overcome by eliciting a second irritant stimulation which detracts from the first pain. Psychophysical studies show that high doses of topical menthol (40%) produce feelings of pain, including pinprick sensations and punctate hyperalgesia (Green, 1985; Hatem et al., 2006; Namer et al., 2005; Wasner et al., 2004). Therefore it has been suggested that topical menthol produces analgesia by acting as a counter-irritant, presumably by noxious event-induced activation of descending inhibitory pathways from the brain, which are known to be activated by noxious stimulation (Le Bars et al., 1979a,b). However, the results reported here argue against this, as analgesic effects were observed with much lower concentrations of menthol (4 mM menthol, which is equivalent to a 0.6% solution) than those reported to produce pain. Additionally, topical icilin was observed to produce analgesia at low doses, but hyperalgesia at high doses, which argues against a mechanism of counter-irritation, where increasing doses would be expected to continually increase the analgesic effect.

5.14 TRPM8-induced analgesia: potential for clinical use

TRPM8 activation stimulates a natural mechanism of the nervous system to suppress pain signalling. This analgesia is effective in a variety of sensitised pain states and is specific to sensitised responses. TRPM8 is therefore a promising target for novel analgesic therapies. TRPM8 agonists could be used clinically to exploit this endogenous mechanism of analgesia without affecting normal acute pain sensation. The effectiveness of topical TRPM8 activators is notable, as it would allow a non-invasive therapy, and would reduce possible side effects associated with central administration of drugs or their vehicles. Although intrathecal administration of agonists was also shown to be analgesic, this is a far more invasive procedure, and would therefore be less desirable for clinical use. The Group II/III mGluRs are another potential target for clinical therapies; however, these receptors are implicated in a variety of functions and dysfunctions in the central nervous system, including motor control, anxiety and depression, learning and memory, and epileptic seizures (Conn & Pin, 1997). Therefore Group II/III mGluR agonists might be expected to

have more undesirable side effects than TRPM8 agonists, and are thus a less promising clinical target.

5.14.1 Effectiveness of TRPM8 activators in other pain states

In order to fully assess the potential clinical use of TRPM8 agonists it would be useful in further experiments to investigate the range of conditions in which TRPM8 agonists produce analgesia. It could be useful to investigate the effect of TRPM8 agonists in other models of chronic pain, such as models of bone cancer-induced pain (Honore et al., 2000); postherpetic neuralgia (Fleetwood-Walker et al., 1999); neuropathic pain produced by chemotherapy drugs, and other models of inflammatory pain. However, the experiments reported here have revealed a significant analgesic effect of icilin in a number of sensitised pain states: neuropathic, demyelination-induced, inflammatory, NMDA-sensitised and cinnamaldehyde-sensitised, suggesting that TRPM8 agonists will be effective in many sensitised pain states. Therefore it may be more beneficial to concentrate on the mechanism of analgesia produced by local cutaneous cooling.

In addition to the inhibitory role in sensitised pain states demonstrated here, previous work has identified icilin, menthol and cooling as effective anti-pruritic agents. Both topical menthol (1%) and cooling the skin (from 32 to 29°C) reduced the intensity of itch induced by the histamine skin-prick test in psychophysical experiments (Bromm et al., 1995). Weaned hairless rats on a low magnesium diet develop pruritus-like symptoms including a rash, and biting and scratching, which are reduced by topical icilin (Wei & Meingassner, 2005). Thus TRPM8 may be a common mediator of relief of both pain and itch, adding to the clinical potential of TRPM8 activators. It would be interesting to investigate animal models of itch to see whether the relief of itch by icilin and menthol is mediated through a similar pathway, perhaps also involving inhibitory glutamate receptors. Itch and pain are mediated through anatomically distinct primary afferent (Schmelz et al., 1997) and spinal cord pathways (Andrew & Craig, 2001), but frequently involve similar molecular mediators (such as histamine and bradykinin activation) and mechanisms (such as central sensitisation of dorsal horn neurons) (Ikoma et al., 2006). It would

therefore be interesting to know to what extent TRPM8 activation relieves itch and to what extent it operates through similar spinal mechanisms.

5.14.2 Alternative TRPM8 activating compounds

This study used menthol and icilin, which are the most well-studied TRPM8 agonists and among the most potent and efficacious (Behrendt et al., 2004). However, there are a variety of other chemical agonists of TRPM8. Many of these are based on the structure of menthol and were developed many years before the discovery of TRPM8, for the property of producing a cooling sensation when applied to the skin (Leffingwell, 2006; Watson et al., 1978). A second group of cooling compounds are based on the tetrahydropyrimidine-2-one structure of icilin (Wei, 2003), and a third group of cooling compounds are cyclic α-ketoenamines (Bassoli et al., 2005). Until recently, icilin was the most potent and efficacious agonist of TRPM8. However, the Wilkinson Sword-developed cooling chemical WS-12 appears to have an even higher affinity for TRPM8 than icilin, with an EC_{50} of 39 nM compared with 500 nM for icilin in similar conditions (Beck et al., 2006). It would be beneficial to further characterise the actions of WS-12 at TRPM8 to investigate whether this chemical indeed is an effective and specific agonist of TRPM8. If so, WS-12 could be a valuable tool for further study of TRPM8, and a potential candidate for clinical use as a TRPM8 activator to produce analgesia.

If TRPM8 agonists are to be used clinically it is important that they are of low toxicity and with as few side-effects as possible. Icilin itself fits this description well as it has few known side-effects and a structure which suggests low toxicity (E.T. Wei, personal communication). Although less potent, menthol has been used safely in over-the-counter topical preparations for many years and therefore is another candidate for clinical use. The downside of both of these compounds is that they have agonist properties at receptors other than TRPM8. It is possible that other TRPM8 agonists, possibly including WS-12, have fewer non-TRPM8 actions and therefore could be more suitable for potential clinical use.

5.14.3 Potential effects of TRPM8 agonists in non-target tissues

TRPM8 expression has been shown outside the peripheral sensory nervous system, most notably in prostate and bladder epithelial cells and also in arterial smooth muscle (Stein et al., 2004; Yang et al., 2006; Zhang & Barritt, 2004). In some of these tissues, TRPM8 may have a pathological function, as its expression is upregulated in prostate cancer, and TRPM8 expression appears de novo in primary breast, lung and skin tumours (Tsavaler et al., 2001). The TRPM8 activator menthol induces apoptosis of prostate cancer cells (Zhang & Barritt, 2004), suggesting that this channel may play a role in tumour cell development or survival (Zhang & Barritt, 2004, 2006).

While the effects of TRPM8 activation in such non-nervous tissues are outside the scope of this thesis, they are relevant to the use of TRPM8 agonists in a clinical setting. It would seem to be sensible to avoid tissues outside the nervous system which express TRPM8, when administering TRPM8 agonists, and particularly to avoid cancerous tissue until the role of TRPM8 in tumour development is more clearly known. It is suggested that TRPM8 agonists could play a positive role in tumour therapy, by stimulating apoptosis of tumour cells (Beck et al., 2006), but from a clinical point of view it would still be undesirable to have TRPM8 agonists administered as pain treatments interacting to an unknown extent and with unknown effects at tumour sites. Therefore systemic administration of TRPM8 agonists should be avoided, as this could result in activation of TRPM8 in these non-target areas and thus potential unwanted side effects. Topical TRPM8 activators applied locally to the area of peripheral innervation would avoid such effects, as would intrathecally delivered drugs, which are confined to the spinal cord.

5.15 Further questions regarding the mechanism of TRPM8-mediated analgesia

There are several key remaining questions regarding the mechanism of TRPM8-mediated analgesia, including: the role of modulation of TRPM8 activity by intracellular signalling pathways; the nature of the spinal cord connections that produce inhibition, including whether it is pre- or postsynaptic; and the involvement

of specific subtypes of inhibitory mGluRs and intracellular signalling pathways engaged by mGluRs. These will be discussed in turn below.

5.15.1 Involvement of intracellular signalling pathways modifying TRPM8

A number of interactions of TRPM8 with endogenous signalling molecules have been identified (Huang et al., 2006). The involvement of these in TRPM8-mediated analgesia was beyond the scope of these experiments, especially as many of the molecules which modify TRPM8 are ubiquitous and are therefore difficult to investigate in in vivo experiments. Nevertheless, endogenous modulation of TRPM8 may well impact on the analgesia mediated through this receptor. Indeed, it is possible, as mentioned above, that endogenous chemical agonists could be released under some circumstances in sensitised pain states to activate TRPM8, partially inhibiting the pain signal.

One key endogenous modulator of TRPM8 is $PI(4,5)P_2$, which has been shown to activate TRPM8 and is required for cold and menthol activation of the channel (covered in more detail in introduction; Rohacs et al., 2005; Liu & Qin, 2005). Therefore it would be expected that stimulation of $PI(4,5)P_2$ formation would facilitate the analgesic effect of icilin and menthol, whereas perturbations that result in $PI(4,5)P_2$ depletion would inhibit analgesia. So it is possible that under some circumstances in vivo, a stimulation of $PI(4,5)P_2$ formation could lead to TRPM8 activation or facilitation, producing an inhibition of pain signalling and of sensitised pain states.

In contrast, a number of key inflammatory signalling molecules are associated with downregulation of TRPM8, although the function of these has not been assessed in vivo. Application of the proalgesic agent bradykinin decreases the sensitivity of TRPM8-expressing neurons to cooling and TRPM8 agonists, and this effect is dependent on PKC activity (Linte et al., 2006; Premkumar et al., 2005). Another endogenous pro-inflammatory agent, PGE_2, has similar effects, decreasing the sensitivity of TRPM8-expressing neurons to cooling (Linte et al., 2006). Decreases in extracellular pH, to levels that occur in inflamed tissue, can inhibit the activation of TRPM8 by icilin and cold, but not menthol (Andersson et al., 2004). Thus TRPM8 function may be inhibited in inflammatory pain states as a result of these signalling

effects, although it is by no means certain that the overall effect of inflammation on TRPM8 in vivo is inhibitory – it is possible that other signalling pathways are activated which result in facilitation of TRPM8. However, an inhibition of TRPM8 in inflammatory pain states could have two alternative consequences. One is that peripherally applied TRPM8 activators might produce a weaker analgesic effect, due to inhibition of TRPM8 by inflammatory mediators. Conversely, it could be true that exogenous application of TRPM8 agonists is particularly helpful in such states, by countering the natural inhibition of TRPM8 function produced by inflammatory mediators.

It is likely that in the future, more functional interactions of TRPM8 with adapter or signalling molecules will be described. It is possible that, in sensitised pain states, one or more signalling pathways results in facilitation of the TRPM8 receptor, so that it is more easily activated. Facilitation could occur at centrally located TRPM8 receptors or at peripherally located receptors, or both, and could explain the apparent selectivity of TRPM8-induced analgesia to sensitised pain states. Facilitation of TRPM8 would lead to increased glutamate release by TRPM8-expressing fibres and thus to an increased inhibition of nociceptive signalling through mGluRs. Such effects could explain the selectivity we have observed with icilin and menthol-induced analgesia.

5.15.2 Further questions regarding the mechanism of spinal cord inhibition

The results in this thesis led to the suggested schematic shown in Figure 5.1, in which activation of TRPM8-expressing afferents causes release of glutamate, which activates Group II/III mGluRs located presynaptically on the terminals of nociceptive afferents or postsynaptically on dorsal horn neurons. However, the precise spinal circuitry underlying this inhibition remains unknown.

Group II mGluRs are pre and postsynaptically located in the spinal cord. One study indicated that they were predominantly presynaptic on afferent terminals in superficial laminae (as shown by rhizotomy) and located on intrinsic spinal neurons in deeper laminae (Carlton et al., 2001), but another study suggested some localisation on spinal cord neurons throughout the cord (Jia et al., 1999), suggesting that they are found both pre and postsynaptically in the spinal cord. They may be

expressed on spinal cord neurons at postsynaptic terminals, or on the presynaptic terminals of spinal interneurons (Jia et al., 1999). Group II mGluR2/3 immunoreactivity is also shown by spinal cord astrocytes (Silva et al., 1999). Electron microscopy shows a predominantly extrasynaptic location at neuron terminals, suggesting a heterosynaptic function (Azkue et al., 2000). Of the Group III mGluRs, mGluR7 appears to be found primarily at presynaptic afferent terminals in superficial laminae, as immunostaining is removed by rhizotomy, (Kinoshita et al., 1998, Li et al., 1997; Ohishi et al., 1995a,b). However, some immunoreactivity remains following rhizotomy, and low levels of mGluR7 mRNA are found in the spinal cord, suggesting some expression by spinal neurons as well (Berthele et al., 1999; Boxall et al., 1998; Neugebauer et al,. 2002; Valerio et al., 1997). mGluR4 is also expressed in the dorsal horn, with dense staining observed in the superficial laminae, localised predominantly in presynaptic terminals (Azkue et al., 2001).

Broadly speaking, the expression pattern of Group II/III mGluRs is consistent with a presynaptic or postsynaptic inhibitory mechanism, and the studies described in this thesis do not distinguish between such mechanisms. It might be possible to elucidate more information about the mechanism of icilin-induced anti-sensitisation analgesia and whether it is pre or postsynaptically mediated, from further electrophysiological experiments. In particular, in preparations such as patch-recordings in spinal cord slice preparations, the contribution of individual inputs and outputs can be more closely examined than in in vivo extracellular recordings. Various electrophysiological phenomena are used to distinguish pre and postsynaptic mechanisms. Effects of a drug on the latency of monosynaptic EPSCs, or effects on the number of transmission failures (failure of a presynaptic action potential to evoke a postsynaptic current) are generally taken as evidence of a presynaptic action (Engelman & MacDermott, 2004). An alteration in the frequency of mEPSCs (miniature EPSCs, which are produced in the absence of action potentials) without a change in their amplitude, is also generally accepted as being due to presynaptic effects (Engelman & MacDermott, 2004; Tsuzuki et al., 2004). These experiments could be used to distinguish between a pre or postsynaptically-mediated mGluR effect in the mechanism of TRPM8-mediated analgesia.

5.15.3 Presynaptically-mediated inhibition

Activation of presynaptic mGluRs is consistent with a potential inhibition of nociceptive transmission, as both Group II and Group III are expressed at high levels by nociceptive afferents. Group II mGluRs are found at high levels in afferent neurons expressing the nociceptive marker IB-4, and in the dorsal horn, staining is particularly high in inner LII, which is predominantly innervated by nociceptive afferents (Carlton et al., 2001). Similarly, mGluR7 is found at high levels in small-diameter DRG neurons, and approximately half of mGluR7-labelled afferents also express the nociceptive markers IB-4 and SP (Li et al., 1997). mGluR4 protein is seen at high levels in small-diameter DRG neurons, and in LII, consistent with expression on the presynaptic terminals of nociceptors (Azkue et al., 2001). Nociceptive afferents terminate predominantly in the superficial dorsal horn and can activate dorsal horn neurons in both superficial laminae and, via an interneuronal connection, neurons in deeper laminae (Basbaum & Jessell, 2000). TRPM8-expressing afferents could activate mGluRs on the presynaptic terminals of nociceptive afferents in the superficial dorsal horn, to inhibit the release of glutamate from the nociceptive afferents. This would decrease the activity of dorsal horn neurons driven by the nociceptive afferents, including those in both superficial and deeper laminae. This is consistent with electrophysiological recordings showing that icilin inhibited the firing of WDR neurons in both superficial and deeper laminae.

If the mechanism of mGluR-mediated inhibition is presynaptic, it could operate either directly or indirectly. As illustrated in Figure 5.2a, TRPM8-expressing cold-sensitive afferents could release glutamate which activates the terminals of neighbouring nociceptive afferents in a heterosynaptic inhibitory mechanism. In a heterosynaptic mechanism, the neurotransmitter is released from a different neuron to the neuron on whose terminal it acts, in contrast to homosynaptic inhibition, in which neurotransmitter released from one neuron acts on receptors located on the terminal of the releasing neuron itself. Both nociceptive afferents and TRPM8-expressing afferents terminate primarily in superficial laminae (Basbaum & Jessell, 2000; Results section 4.5.4), and therefore the terminals could be anatomically close enough to allow such an interaction. Electron microscopy studies show that a large proportion of Group II mGluR expression is extrasynaptic, rather than at the

presynaptic terminal itself, which suggests a heterosynaptic mechanism of activation for these receptors (Azkue et al., 2000), and heterosynaptic activation of mGluRs has been demonstrated in other areas of the nervous system, such as the cerebellum and the hippocampus (Mitchell & Silver, 2000; Scanziani et al., 1997). The inhibition of glutamate release from presynaptic afferent terminals would lead to inhibition of downstream dorsal horn neurons.

Alternatively the mechanism could operate through interneurons (as shown in Figure 5.2b). Glutamate released from TRPM8-expressing afferents could activate glutamate-releasing interneurons, which in turn synapse onto the terminals of nociceptive primary afferents to activate inhibitory glutamate receptors. Such glutamatergic interneurons synapsing onto primary afferent terminals have been shown in immunohistochemical studies (Vesselkin et al., 2003).

An interneuronally-mediated mechanism resembles the gate control theory of Melzack and Wall (Melzack & Wall, 1965). In this theory, large Aβ fibre mechanoreceptors were proposed to produce inhibition of nociceptive fibres via interneurons which synapse onto the nociceptor terminals to inhibit firing (see Introduction Figure 1.2). The suggestion outlined in this thesis is that input from cooling-sensitive afferents, which could be either C or Aδ fibres, produces an inhibitory gate control over nociceptive populations. The original theory had Aβ fibres exerting the inhibitory gate, rather than C or Aδ fibres. However, it was not crucial to the theory that the inhibitory neurons were Aβ fibres, only that they were a different group of afferents to the nociceptive afferents, and produced inhibition of the nociceptive afferents. There is evidence for presynaptic inhibition of nociceptive afferents produced by activity in other small-diameter afferents: one study showed that repetitive activation of sciatic Aδ fibres produced a presynaptically-mediated inhibition of saphenous C afferents (Jones et al., 2005), and Aδ fibre stimulation has been shown to produce a long-term depression of C fibre-evoked spinal field potentials (Liu et al., 1998).

A second difference from the original gate control theory is that the interneurons proposed by Melzack & Wall were suggested to be inhibitory GABAergic neurons, rather than glutamatergic neurons. However, the importance of these neurons is that they are functionally inhibitory, regardless of the transmitter released. Therefore it is

possible that glutamatergic interneurons activated by TRPM8-expressing afferents could mediate an inhibitory gate over nociceptive information and thereby explain the results found here.

5.15.4 Postsynaptically-mediated inhibition

It is possible that TRPM8-expressing afferents could also activate postsynaptically located Group II/III mGluRs, reducing the likelihood of postsynaptic excitation and thereby reducing transmission of nociceptive information through the spinal cord. A postsynaptic mechanism could account for the inhibitory effect of icilin on NMDA-mediated hypersensitivity, which, as described above, is thought to be mainly postsynaptically mediated. We saw inhibitory effects of icilin on WDR neurons in deeper laminae. This could be mediated postsynaptically by an inhibition of excitatory interneurons that relay signals from nociceptive afferents to deep WDR neurons, or by the TRPM8-mediated activation of an inhibitory interneuronal pathway that terminates on the deep WDR neurons. There are two apparent problems with a postsynaptically mediated mechanism. Firstly, glutamate acting on postsynaptic terminals would be expected to have a mainly excitatory effect, due to the high prevalence of AMPARs and NMDARs on postsynaptic terminals, and it is not clear how an inhibitory Group II/III mGluR-mediated effect could be selectively produced by glutamate acting on postsynaptic neurons. Secondly, the majority of Group III mGluR localisation is thought to be presynaptic on afferent terminals (Azkue et al., 2001; Ohishi et al., 1995a,b). Therefore it is unclear to what extent a postsynaptic mechanism could operate.

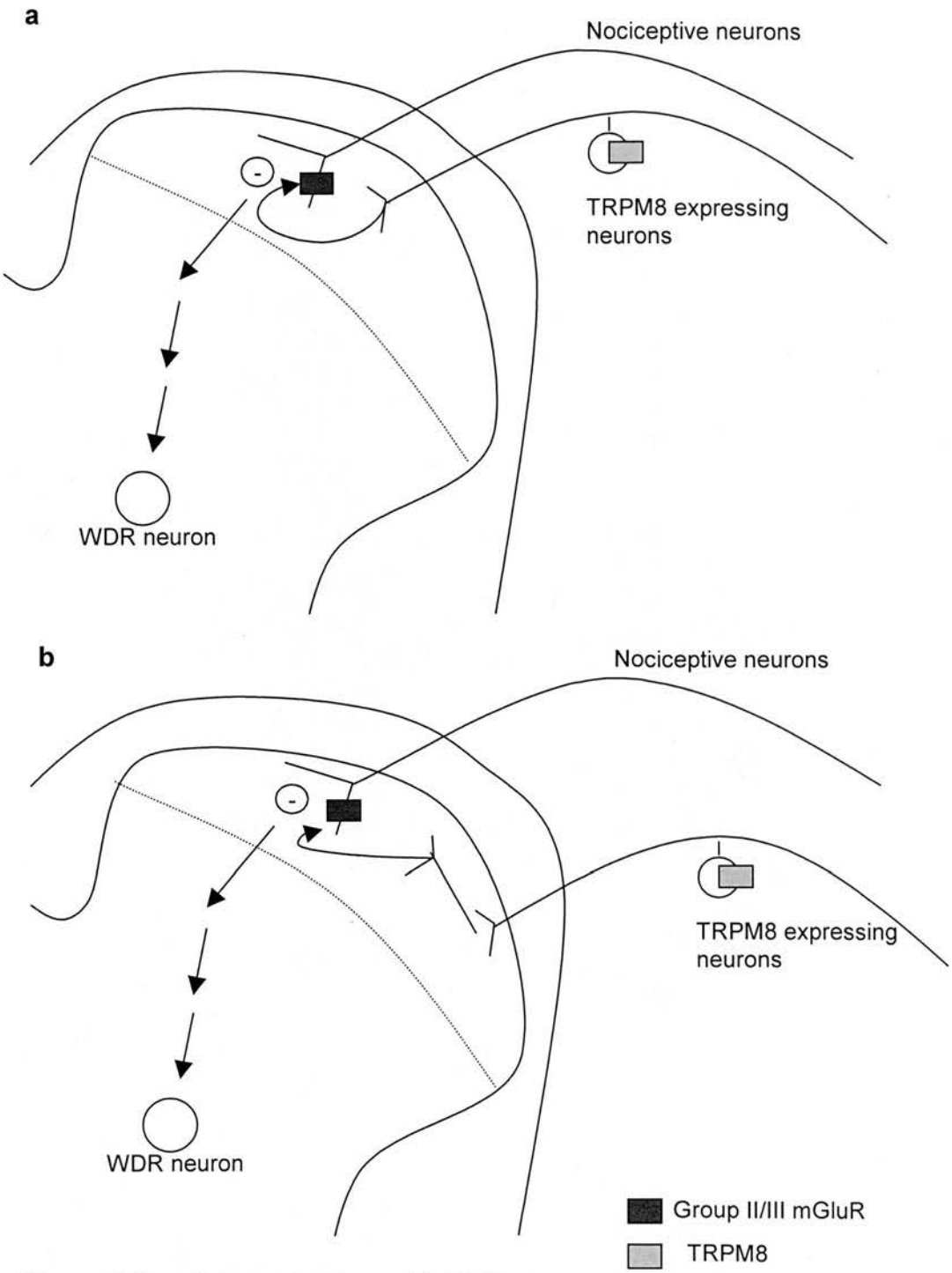


Figure 5.2 Possible mechanisms of inhibition
a) Activation of TRPM8-expressing cool-sensitive non-nociceptive afferents releases glutamate, which then acts through inhibitory mGluRs located on the presynaptic terminals of nociceptive afferents in superficial laminae. This consequently results in inhibition of the WDR dorsal horn neurons driven by the nociceptive afferents, including those in deeper laminae contacted by an interneuronal pathway.
b) TRPM8-expressing neurons release glutamate to activate interneurons which in turn feed back onto nociceptive terminals, activating inhibitory mGluRs. This then inhibits the nociceptive afferent-driven activation of dorsal horn WDR neurons.

5.16 Overall conclusions

In summary, the experiments in this thesis have demonstrated a common mechanism underlying the phenomenon of analgesia produced by cutaneous cooling and the phenomenon of analgesia produced by menthol and mint oils. Study of the function of TRPM8 in vivo has shown that peripheral or central activation of TRPM8 produces analgesia which is effective in chronic pain states, including neuropathic pain, and is dependent on Group II/III mGluRs. Therefore TRPM8 is indicated as a novel target for analgesic therapies for use in chronic pain states. Group II/III mGluRs could also be potential targets for analgesic drugs, although would be more likely to produce additional side effects. These results demonstrate a phenomenon in which activation of one class of sensory afferent inhibits transmission from nociceptive afferents through a central mechanism. This work points the way to further research, both to develop TRPM8 activators as a strategy for clinical treatment of pain, and to elucidate the spinal cord circuitry which underlies TRPM8-mediated analgesia.

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Appendix

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Article

Analgesia Mediated by the TRPM8 Cold Receptor in Chronic Neuropathic Pain

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Summary

Background: Chronic established pain, especially that following nerve injury, is difficult to treat and represents a largely unmet therapeutic need. New insights are urgently required, and we reasoned that endogenous processes such as cooling-induced analgesia may point the way to novel strategies for intervention. Molecular receptors for cooling have been identified in sensory nerves, and we demonstrate here how activation of one of these, TRPM8, produces profound, mechanistically novel analgesia in chronic pain states.

Results: We show that activation of TRPM8 in a subpopulation of sensory afferents (by either cutaneous or intrathecal application of specific pharmacological agents or by modest cooling) elicits analgesia in neuropathic and other chronic pain models in rats, thereby inhibiting the characteristic sensitization of dorsal-horn neurons and behavioral-reflex facilitation. TRPM8 expression was increased in a subset of sensory neurons after nerve injury. The essential role of TRPM8 in suppression of sensitized pain responses was corroborated by specific knockdown of its expression after intrathecal application of an antisense oligonucleotide. We further show that the analgesic effect of TRPM8 activation is centrally mediated and relies on Group II/III metabotropic glutamate receptors (mGluRs), but not opioid receptors. We propose a scheme in which Group II/III mGluRs would respond to glutamate released from TRPM8-containing afferents to exert an inhibitory gate control over nociceptive inputs.

Conclusions: TRPM8 and its central downstream mediators, as elements of endogenous-cooling-induced analgesia, represent a novel analgesic axis that can be exploited in chronic sensitized pain states.

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Introduction

Chronic neuropathic pain arising from peripheral nerve damage is a severe clinical problem with limited treatment options [1]. Changes in both damaged and undamaged primary afferent neurons as well as central (spinal cord) sensitization lead to hyperalgesia (accentuated responses to painful stimuli), allodynia (pain in response to normally innocuous stimuli), and spontaneous pain. We hypothesized that elucidating the poorly understood mechanisms underlying cold-induced analgesia might lead the way to novel neuropathic analgesics.

Since Hippocrates and Galen [2, 3], sporadic reports have described the use of cooling to produce analgesia [4]. Clinical trials show beneficial effects of cooling on chronic back pain, dental pain, postoperative pain, and muscle injuries [5]. Preparations containing menthol, which produces a cool sensation, are used topically to relieve neuralgia in traditional Chinese and European medicine [6, 7]. Mint oil has been reported to alleviate thermally elicited pain and postherpetic neuralgia [8, 9], and oral menthol can cause short-term analgesia [10]. Furthermore, in mice, oral or intracerebroventricular application of menthol decreased nociceptive responses to the hot-plate test and acetic-acid writhing test [11]. Despite this history, no definitive mechanism has been established for cool-induced analgesia.

The recent isolation of the transient receptor potential (TRP) cation channels present in primary sensory neurons has revolutionized our understanding of cutaneous temperature detection. The best-characterized example is the capsaicin- and heat-sensitive TRPV1 receptor [12], and although much less is known about cool-sensitive TRPs, they are the target of intensive research [13]. TRPM8 is activated at innocuous cool temperatures (with 50% activation around 18°C–19°C [14]) and by menthol and icilin [15, 16], which act as selective (but not totally specific) activators of the channel [14, 17]. The TRPM8 channel is expressed by a subpopulation of sensory neurons in dorsal root ganglia (DRG) and trigeminal ganglia [15, 16], where responses to cooling correlate well with mRNA expression and menthol sensitivity [18–20].

The TRPA1 channel is also expressed in DRG and trigeminal ganglia [14, 21] and is reportedly activated by cooling temperatures beginning 5°C–6°C lower than that for TRPM8 [14, 22] and by noxious chemicals such as cinnamaldehyde and mustard oil [14, 22, 23]. However, the role of TRPA1 in physiological cold sensation is currently unclear [13], with some reports of TRPA1 not being activated by cold [21, 23] and of normal cold sensitivity in TRPA1 knockout mice [24]. In contrast, another study of TRPA1 knockout mice reported attenuated responses to noxious cold [25], and antisense knockdown studies show a decrease in development of nerve-injury- or inflammation-induced hyperalgesia to intense cold stimuli [26, 27]. On balance, TRPA1 seems a less likely candidate than TRPM8 for the mediator of cooling-induced analgesia.

A central spinal mechanism of analgesia is suggested, because cutaneous cooling can prevent pain produced by afferent stimulation [4]. Glutamate-receptor-dependent plasticity in spinal cord neurons commonly underlies chronic pain states with both ionotropic and metabotropic receptors participating at pre- and postsynaptic sites. Although most glutamate receptors are excitatory, the Group II/III metabotropic receptor (mGluR) subtypes exert inhibitory influences, suggesting the hypothesis that they could potentially underpin cooling-induced analgesia. Indeed, Group II and III mGluRs are present in the spinal cord largely on afferent terminals, but with some glial and postsynaptic expression [28, 29], and their activation inhibits both nerve-injury- and inflammation-induced sensitization of neuronal and behavioral responses [30–32].

Here we demonstrate marked analgesic effects of peripherally or centrally applied TRPM8 activators (such as icilin or menthol), or mild cooling of the skin, in a model of neuropathic pain. TRPM8 levels in DRG and superficial dorsal horn were increased after nerve injury. Analgesia was restricted to injury-sensitized responses and abolished after antisense knockdown of TRPM8. Peripheral application of icilin or menthol also activated slowly conducting afferents (in a TRPM8-antisense-sensitive manner) and suppressed the increased responsiveness of single dorsal-horn neurons ipsilateral to nerve injury. In contrast, activation of TRPA1 produced hyperalgesia (in both naive animals and nerve-injured animals). TRPM8-mediated analgesia was selectively reversed by intrathecal administration of Group II/III mGluR antagonists and mimicked by agonists, and iontophoresis of a Group III mGluR antagonist, as an example, reversed the inhibitory effect of icilin on sensitized single neurons. Sensitization-specific analgesia from TRPM8 activation was also observed in inflammatory afferent demyelination and TRPA1 activator-induced pain models.

Results

Reversal of Nerve-Injury-Induced Reflex Sensitization by Peripheral Activation of TRPM8 Channels

In order to model potential clinical usage, we administered icilin topically to the paws by placing rats with chronic constriction injury (CCI) to sciatic nerve in a bath with 1-cm-deep drug solution, kept at 30°C to avoid any effects on local skin temperature. After 5 min, icilin (80 μM), but not vehicle (0.2% dimethylformamide in water), caused striking reversal of CCI-induced behavioral-reflex sensitization to thermal and mechanical stimuli (Figure 1A). Concentration-dependent effects were observed from 2.5 μM up to a maximum of 500 μM, with no effect on contralateral or naive responses (Figure 1B). At much higher concentrations of icilin, we observed the beginning of a trend toward increasing reflex sensitivity, ipsilateral and contralateral to CCI and in naive animals (Table 1), that was statistically significant in mechanical and (after a delay) in thermal tests, only at the highest concentration tested, 5 mM. This effect was distinct from the prominent analgesia at low concentrations of icilin because it was not specific to a sensitized state and may be due to weak interaction with other targets or nonspecific actions. Specific reversal of sensitized responses was also caused by another selective TRPM8 activator, (−)-menthol (4 mM). The stereoisomers isomenthol and (+)-menthol, which are several-fold-less-potent agonists of TRPM8 [33, 34], also produced reversal of sensitized responses at concentrations of 8 mM and 16 mM (Figure 1C).

Icilin is expected to activate TRPM8-containing afferents, so we recorded firing activity in saphenous-nerve afferents after topical application of icilin (Figure 1D). The nerve was dissected to produce small-number preparations of fine afferents, with conduction velocities of up to 2.6 m/s (representing C- and Aδ-fiber afferents [35]). Icilin (200 μM) applied to the receptive field on the hind limb caused a significant increase in firing frequency in 21.6% (40 out of 185) of recorded fine afferents with a mean 7-fold increase in firing frequency from baseline of 4.5 ± 2.5 Hz to 31.6 ± 3.4 Hz and a mean time to peak effect of 3.3 ± 0.5 min. Icilin did not produce rapid desensitization, agreeing with some reports [36] but not others [37]. Recovery was consistently observed. Similar results were obtained from both hairy and glabrous skin. Large myelinated mechanoreceptors (conduction velocities 6.8–15 m/s, n = 43) were unaffected.

Consistent with a TRPM8-mediated mechanism, paw immersion at 16°C–20°C for 5 min also produced statistically significant mechanical analgesia (Figure 1E). Recordings from a subcutaneous thermistor probe showed that deep skin temperatures were 0.5°C above bath temperatures after 5 min in similar conditions. This temperature is in the range expected to activate TRPM8 and stimulate innocuous cool-sensitive fibers. Immersion temperatures below 14°C (in the range where other cold sensors, in addition to TRPM8, are also likely to be activated) elicited active nociceptive withdrawal reflexes limited to the period of hind paw immersion, in agreement with the known temperature activation range for nociceptive cold fibers [38].

Localization of TRPM8 in Afferents and Superficial Dorsal Horn: Increased Expression after Nerve Injury

The presence and localization of TRPM8 in DRG and the spinal cord were investigated by immunoblotting and immunohistochemistry. After rapid homogenization of DRG in Laemmli lysis buffer and SDS-PAGE, immunoblots probed with a rabbit polyclonal antibody raised to TRPM8 residues 278–292 and 1090–1104 (human) [39] showed a single, strong band at approximately 128 kDa (the predicted molecular weight of TRPM8), with faint bands observed at approximately 170, 60, and 50 kDa (Figure 2A). Both antigen-preabsorbtion and antisense-knockdown controls were consistent with specificity of this antibody in recognition of TRPM8 at approximately 128 kDa under the conditions used. Preincubation of the antibody with membranes from COS7 cells transfected with TRPM8 expression plasmid abolished the band at 128 kDa, whereas sham preabsorbtion with membranes from cells with empty vector did not (Figure 2A). Correspondingly, intrathecal delivery of a TRPM8 antisense oligonucleotide to naive (non-CCI) rats over 5 days also resulted in almost complete knockdown of the 128 kDa band (Figure 2A), whereas a

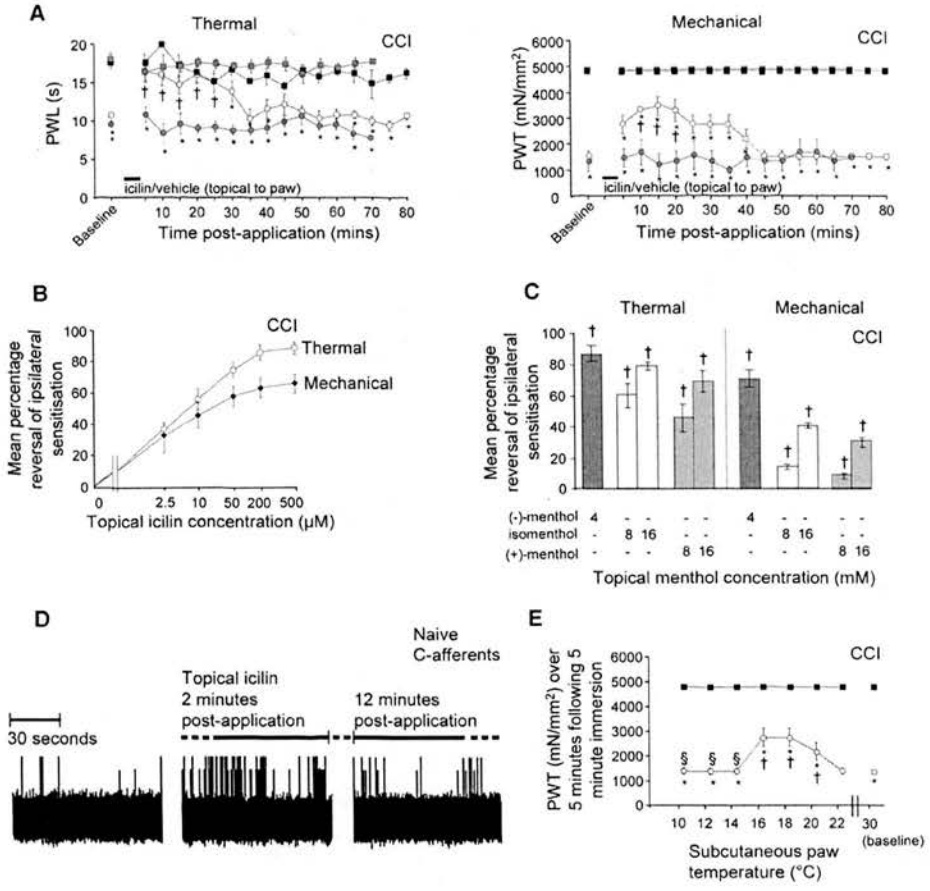

Figure 1. Peripheral TRPM8 Activation and Moderate Cooling Are Analgesic after CCI

(A, B, C, and E) Behavioral data from CCI animals, shown as mean ± SEM; each graph represents n of six animals.

(A) Paw withdrawal latency (PWL; s) to noxious heat and paw withdrawal threshold (PWT; mN/mm^2) to mechanical stimuli before and after 5 min paw immersion in a shallow 30°C water bath containing 80 μM icilin or vehicle. ○: ipsilateral paw plus icilin; ●: ipsilateral + vehicle; ■: contralateral plus icilin, ▩: contralateral plus vehicle. * indicates significant ipsilateral-contralateral differences; † indicates significant difference from predrug baseline ($p < 0.05$).

(B) Concentration-response curve for mean ± SEM percentage reversal of ipsilateral sensitization for thermal (○) or mechanical (◆) tests calculated over 10–15 min after paw immersion in 2.5–500 μM icilin.

(C) Reversal of ipsilateral sensitization by paw immersion in (−)-menthol at 4 mM, and by higher concentrations of the less-potent stereoisomers (+)-menthol and isomenthol (8 and 16 mM). Values were calculated over 10–25 min after paw immersion from experiments as shown in (A). † indicates significant differences from predrug baseline ($p < 0.05$).

(D) Typical electrophysiological recording from the subpopulation of icilin-responsive C fiber afferent fibers before, 2 min after topical application of icilin to the receptive field (peak effect), and 12 min later.

(E) Mechanical analgesia measured over 5 min after 5 min immersion of paws at the indicated temperature range. § denotes spontaneous withdrawal responses during the immersion period, * denotes significant difference from contralateral paw, and † denotes significant difference from preimmersion baseline ($p < 0.05$).

missense control oligonucleotide was ineffective (see below). Intrathecally delivered fluorescent-labeled oligonucleotides have been shown to effectively penetrate the DRG, as soon as 4 hr after initial delivery [40]. The faint bands, at 50 and 60 kDa at least, remained present in each case and so are likely to represent nonspecific interactions of the antibody under these conditions. As further controls, we showed that TRPV1 immunoreactivity was unaffected by treatment with the TRPM8 antisense reagent and that the housekeeping enzyme GAPDH (36 kDa) was evenly present in each lane (Figure 2A). After nerve injury, there was a marked increase in expression of the 128 kDa TRPM8-immunoreactive band specifically in ipsilateral, but not contralateral, DRG (Figure 2A), whereas immunoreactivity for GAPDH was unaltered. Densitometric ratios for TRPM8 expression as percentage of GAPDH were 80.7 ± 4.1 ipsilateral to CCI, which was significantly greater than that seen contralateral to CCI (49.3 ± 3.2) and in naive DRG (50.7 ± 2.7) (mean ± standard error of the mean [SEM], n = 5–6). L4–5 spinal cord extracts showed that TRPM8 immunoreactivity was present centrally, and after preparation of a crude particulate fraction (centrifugation at 11,000 × g for 45 min), these also showed consistent increases in expression ipsilateral to injury. Densitometric values ipsilateral to CCI were 198% ± 6.7% of those from naive tissue ($p < 0.05$; mean ± SEM, n = 5), whereas contralateral values were 125% ± 7.1% ($p > 0.05$).

Table 1. Analgesic Effects of Topically Administered Icilin against Neuropathic Sensitization Revert at Very High Concentrations to a General Nociceptive Effect

Reflex Response Sensitivity at Different Times after Icilin Application to Paw						
Thermal PWL (s)						
Drug Concentration (μM)	Naive		CCI Ipsi		CCI Con	
Predrug Baseline	15.5 ± 0.2		9.7 ± 0.2		16.4 ± 0.2	
	15 min	50 min	15 min	50 min	15 min	50 min
0 (vehicle)	15.1 ± 1.2	14.9 ± 0.8	9.8 ± 1.1	9.7 ± 0.9	15.2 ± 0.6	15.1 ± 0.7
1000	15.1 ± 1.2	14.8 ± 0.9	16.4 ± 0.8[a]	8.6 ± 0.2	16.1 ± 0.5	15.2 ± 0.4
2500	14.9 ± 0.3	13.2 ± 0.8	16.4 ± 0.9[a]	8.4 ± 0.6	16.8 ± 0.9	15.3 ± 0.7
5000	15.3 ± 0.5	13.1 ± 0.7[b]	16.7 ± 1.0[a]	7.2 ± 0.6[b]	16.1 ± 0.9	13.4 ± 0.7[b]
Mechanical PWT (mN/mm^2)						
Drug Concentration (μM)	Naive		CCI Ipsi		CCI Con	
Predrug Baseline	4830.6 ± 0.0		805.7 ± 26.3		4793.6 ± 37.5	
	15 min	50 min	15 min	50 min	15 min	50 min
0 (vehicle)	4830.6 ± 0.0	4830.6 ± 0.0	805.7 ± 26.3	805.7 ± 26.3	4830.6 ± 0.0	4830.6 ± 0.0
1000	4830.6 ± 0.0	4830.6 ± 0.0	702.8 ± 185.3	702.8 ± 185.3	4530.1 ± 300.5	4530.1 ± 300.5
2500	4830.6 ± 0.0	4830.6 ± 0.0	651.9 ± 193.0	651.9 ± 193.0	4454.9 ± 245.9	4454.9 ± 245.9
5000	3828.6 ± 316.8[b]	4830.6 ± 0.0	412.2 ± 24.3[b]	736.0 ± 26.3	2040.2 ± 115.2[b]	4830.6 ± 0.0

Significant effects of drug on reflex responses are indicated:
[a] Significant increase from baseline, indicating analgesic effect of icilin.
[b] Significant decrease from baseline values, indicating hyperalgesic effects.

Immunohistochemistry was carried out with a rabbit polyclonal antibody raised to TRPM8 residues 656–680 (rat) [41], the specificity of which was addressed by antigen-preabsorbtion and antisense-knockdown controls. The labeling observed in a discrete subpopulation of DRG cells was abolished after preincubation with the peptide antigen (no positive cells seen, counted over twelve 500 μm^2 sections, compared with a mean of 5.3 ± 0.4 TRPM8-positive cells per 500 μm^2 DRG section with sham treatment of antibody, and 5.1 ± 0.5 TRPM8-positive cells per 500 μm^2 DRG section with untreated antibody, counted over 12 sections each). In immunoblots, the antibody also labeled in naive DRG tissue a single band, at approximately 128 kDa, that was abolished either by preabsorbtion with the peptide antigen or by prior 5 day intrathecal infusion of TRPM8 antisense (Figure 2B). Immunohistochemical staining in the spinal cord showed that TRPM8 was largely expressed in the superficial dorsal horn, like the C fiber marker peripherin (Figure 2C), and that after dorsal rhizotomy (L2–6), the vast majority of TRPM8 (and peripherin) immunoreactivity was lost ipsilaterally (reductions of approximately 80%–90%), suggesting that spinal TRPM8 originates largely from afferents. In confirmation of the immunoblot findings, levels of TRPM8-like immunoreactivity were increased in the dorsal horn ipsilateral to injury (by approximately 70%–80%), but retained a similar distribution to that in naive animals (Figure 2D).

To establish whether the increases in afferent TRPM8 expression occurred in specific subpopulations of DRG cells, we investigated TRPM8 colocalization with markers of myelinated afferents (neurofilament-200; NF-200 [42]) and unmyelinated afferents (peripherin [43]). In naive rats, TRPM8 immunoreactivity was largely confined to a subpopulation of unmyelinated DRG cells (8.3% ± 0.2% of peripherin-positive cells; 34 of 408 cells) and only minimally expressed in myelinated, NF-200-positive cells (1.3% ± 0.5%; 6 of 445 cells). However, after CCI, TRPM8 expression was significantly increased ipsilaterally in both NF-200- and peripherin-positive cells, to 7.9% ± 1.2% (31 of 390 cells) and 15.5% ± 0.8% (64 of 412 cells), respectively. Corresponding contralateral values were unaltered from naive values, at 2.0% ± 0.4% (14 of 346 cells) and 9.2% ± 0.4% (42 of 452 cells) (Figures 2E and 2F). Data were taken from 3 CCI and 3 naive animals and counted across 15–21 sections. The additional TRPM8-expressing NF-200-positive cells were small (average diameter, 19.7 ± 0.8 μm), presumed Aδ myelinated neurons [35]. There were no significant differences in the diameters of NF-200- or peripherin-positive cells or in the numbers of NF-200- or peripherin-positive DRG neurons per section.

Molecular Identification of TRPM8 as the Mediator of Icilin-Induced Analgesia

To define the specific involvement of TRPM8 in icilin analgesia, we further utilized the antisense-oligonucleotide knockdown strategy. TRPM8 antisense or mismatched control oligonucleotides were delivered intrathecally over 13 days to parallel the sensitization developing after CCI. The development of CCI-induced behavioral-reflex sensitization was unaffected, including thermal hyperalgesia and mechanical allodynia (Figures 3A and 3B) and cold allodynia (control CCI animals showed elevation of the paw ipsilateral to nerve injury out of 4°C water for 8.1 ± 0.5 s at peak, 9–11 days after surgery, whereas corresponding values in antisense-treated CCI animals were 7.6 ± 0.6 s). In contrast, the reversal of neuropathic reflex sensitization produced normally by 80 μM icilin applied to the paws (Figure 1A) was abolished by treatment with antisense (Figure 3A), but not missense (Figure 3B), reagents. The mean ± SEM reversals of ipsilateral sensitization over 10–25 min after icilin treatment in antisense- and missense-treated animals were 7.7% ± 7.4% and 82.8% ± 6.9%, respectively, for paw withdrawal latency (PWL), and

9.4% ± 8.2% and 58.7% ± 8.2% for paw withdrawal threshold (PWT), with missense-treated, but not antisense-treated, animals retaining significant effects of icilin ($p < 0.05$). When antisense osmotic pumps were depleted, but animals were still neuropathic (18 days after surgery for insertion of 14 day minipumps and CCI), responses to icilin were restored to 83.7% ± 10.1% reversal of sensitization for PWL and 54%.0 ± 7.2% for PWT. Effectiveness of knockdown was assessed by SDS-PAGE/immunoblotting. Expression of the 128 kDa TRPM8-immunoreactive band in both ipsilateral and contralateral DRG was greatly reduced by the antisense reagent, and the increase in TRPM8 expression normally seen ipsilateral to nerve injury was prevented (Figure 3C). The missense reagent had no effect (Figure 3C), showing TRPM8 expression similar to that in untreated CCI animals (Figure 2A). In missense-treated animals, TRPM8:GAPDH ratios were 77.9% ± 2.0% ipsilateral to CCI and 52.9% ± 2.1% contralateral, similar to corresponding control values (Figure 2A and above), whereas in antisense-treated animals, values were much lower (19.8% ± 2.2% and 14.9% ± 2.1%, respectively, mean ± SEM, n = 5).

To confirm that antisense knockdown of TRPM8 resulted in associated functional changes in afferents, we made saphenous-nerve recordings from naive animals receiving intrathecal delivery of TRPM8 antisense or missense oligonucleotides, 4–5 days after insertion of the pump. The increase in firing frequency evoked by topical icilin (200 μM) was strongly reduced in animals receiving antisense. Only 3 out of 34 recorded slowly conducting fibers (8.8%) showed a partial activation in response to the drug, increasing firing by approximately 2-fold from a baseline of 5.8 ± 1.4 to 12.7 ± 0.6 Hz, compared with the 7-fold increase observed in over 20% of fine afferents in naive animals. In contrast, missense animals showed a 7-fold increase in firing frequency in 25% of 40 recorded fibers from a baseline of 3.3 ± 0.7 to 23.1 ± 3.2 Hz, similar to results from naive animals. Similarly, in missense-treated animals, topical (−)-menthol (4 mM) produced an approximately 8-fold increase in mean firing frequency (from 4.5 ± 2.9 to 38.9 ± 7.6 Hz), activating 20% of fibers (35 identified afferents recorded). This compared with no obviously activated afferents in antisense-treated animals (mean firing frequency 4.0 ± 1.8 Hz at background, 4.8 ± 1.9 Hz after drug application, 28 identified afferents recorded). However, TRPM8 antisense treatment did not alter the effect of topically applied resiniferatoxin (1 mM), a potent TRPV1 agonist acting as a control. In antisense-treated animals, resiniferatoxin evoked a 6-fold increase in firing frequency in activated afferents (from 4.5 ± 0.7 baseline to 25.9 ± 1.8 Hz at peak response, 16 afferents activated out of 28 recorded), which was similar to responses in missense-treated animals (showing a mean 5-fold change in firing frequency from 4.6 ± 2.8 to 24.8 ± 3.1 Hz).

Central Intrathecal Administration of TRPM8 Activators Also Inhibits Neuropathic Sensitization

Because TRPM8 is present on central terminals of primary sensory neurons as well as their peripheral terminals (Figures 2B and 2C, [44, 45]), we investigated whether intrathecal application of TRPM8 activators near the central terminals would also produce analgesia. Intrathecal injection of icilin (10 nmol) produced robust reversal of CCI-induced behavioral-reflex sensitization in thermal and mechanical tests (Figure 4A, $p < 0.05$ for up to 55 min). Intrathecal injection of (−)-menthol (200 nmol) in CCI rats also caused a significant reversal of the sensitized responses, lasting 35–40 min (Figure 4B). Because of the higher potency and efficacy of icilin at TRPM8 [15], further experiments mainly utilized icilin as the representative TRPM8 activator. Icilin produced dose-dependent analgesic effects restricted to the nerve injury side in both thermal and mechanical tests that were statistically significant by 0.125 nmol and increased to almost complete reversal of sensitization by 10 nmol. Nonlinear curve-fitting indicated that maximal effects of icilin were similar for PWL and PWT (91.6% ± 9.9% and 82.6% ± 6.8% reversal of sensitization, respectively), as were EC_{50} values (dose for 50% of maximal effect; 0.17 ± 0.02 nmol and 0.31 ± 0.02 nmol, respectively).

In complete contrast to the effects of TRPM8 activators, the TRPA1 activator, cinnamaldehyde [22] (75 nmol injected intrathecally), significantly increased reflex responsiveness in thermal and mechanical tests and was effective contralateral as well as ipsilateral to nerve injury (Figure 4C). The sensitizing effects of cinnamaldehyde were prevented by coinjection of Ruthenium Red (0.25 nmol), which can block TRPA1 channels [21, 46], whereas the analgesic effect of intrathecally injected icilin was unaffected (Figure 4D). Sensitizing effects of cinnamaldehyde were also seen in naive animals, with a 36.9% ± 7.9% reduction in PWL and a 41.9% ± 6.7% reduction in PWT. Similar effects were produced by two further TRPA1 activators [46, 47], allicin (25 nmol), where corresponding reductions were 33.2% ± 5.9% and 20.7% ± 8.2%, respectively, and diallyl disulphide (50 nmol), with equivalent values of 25.9% ± 7.0% and 28.5% ± 6.2% (mean ± SEM, n = 3–6). In contrast, the TRPM8 activators icilin (10 nmol) and (−)-menthol (200 nmol) were without effect in naive animals (data not shown). Topical application of cinnamaldehyde (1.5 mM) also produced bilateral sensitization of behavioral reflexes in naive animals (mean decrease of 32.0% ± 8.6% in PWL and 20.2% ± 8.2% in PWT, $p < 0.05$, n = 6). This corresponds to the licking and shaking behavior as well as the decrease in PWL reported after intraplantar injection of cinnamaldehyde [22].

In further experiments, we investigated whether the sensitized pain behaviors caused by TRPA1 activators or other pain models were susceptible to icilin-induced analgesia. Sensitization caused by intrathecal or topical cinnamaldehyde was markedly attenuated by intrathecal icilin (Table 2). The effect of topical cinnamaldehyde was additionally reversed by topical icilin (200 μM, data not shown). Sensitization caused by focal demyelination of the sciatic nerve [43] or intraplantar injection of Complete Freund's Adjuvant (CFA) was also significantly inhibited by intrathecal icilin (Table 2).

Central Mechanism of Icilin Reversal of Neuropathic Sensitization Involves mGlu Group II/III Receptors

Because topical icilin increases activity in fine afferents (Figure 1D) and both intrathecal and topical icilin reverse nerve-injury-induced sensitization, centrally mediated

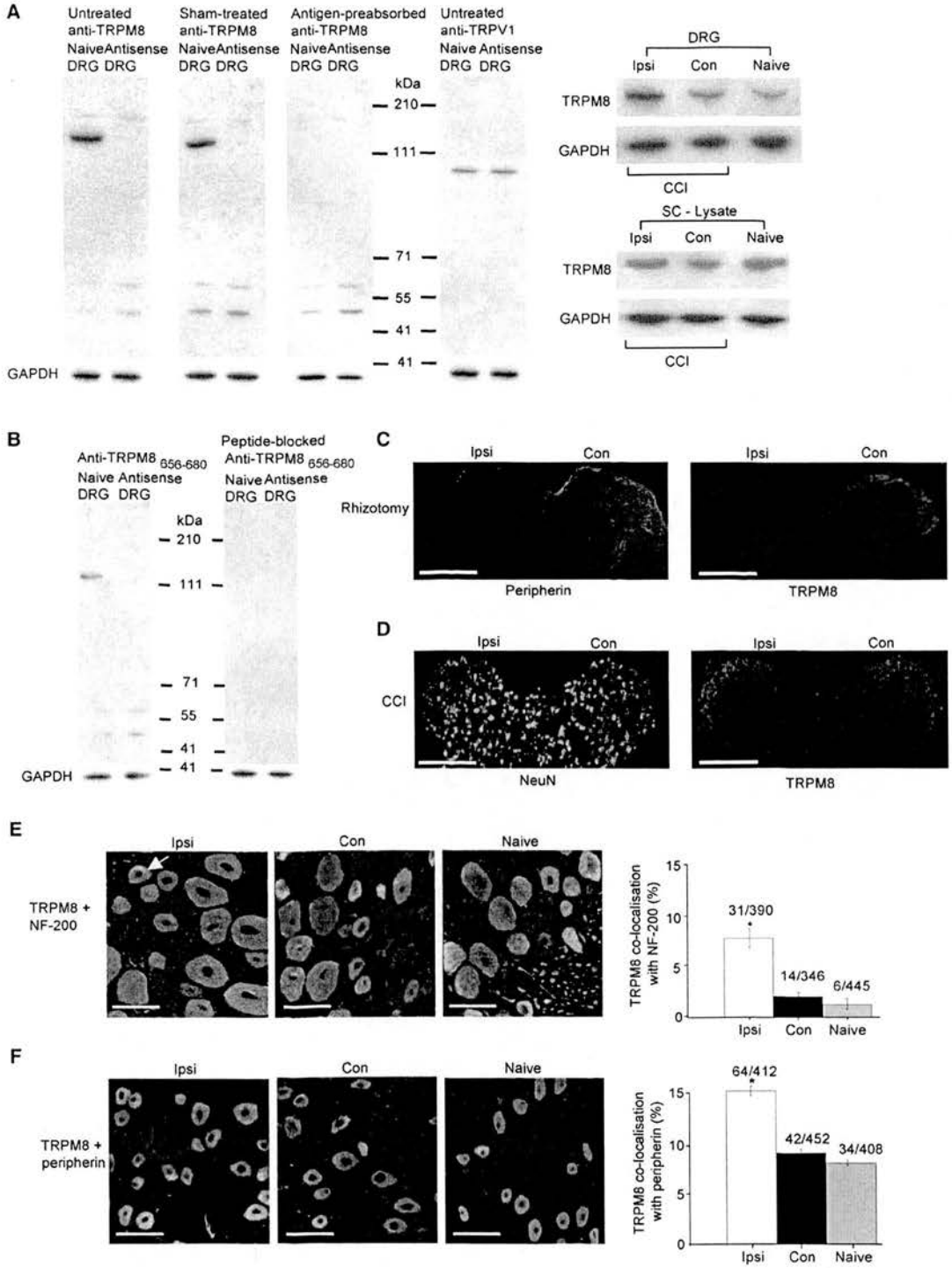

Figure 2. TRPM8 Immunoreactivity Is Present in DRG and Spinal Cord, Arises from Afferents, and Is Increased Ipsilateral to CCI

(A) Immunoblots of DRG show whole gels with TRPM8 protein running at 128 kDa and additional faint bands at approximately 170, 60, and 50 kDa, in normal rat DRG with specific knockdown of the 128 kDa band in DRG from antisense-treated animals. In additional controls, when the TRPM8 antibody was preincubated with membranes from TRPM8-expressing cells, the 128 kDa immunoreactive band was removed, whereas sham treatment had no effect. Blots additionally show GAPDH loading controls. TRPV1 expression (single band at ~90 kDa) was unaltered in DRG from TRPM8 antisense-treated animals. Immunoblots for TRPM8 protein showed a clear increase in expression of the specific 128 kDa band in DRG ipsilateral ("ipsi") to nerve injury compared to contralateral ("con") and naive DRG, with no change in GAPDH. Spinal cord (SC) whole lysates showed no discernable changes in TRPM8 levels; however, increased levels ipsilateral to nerve injury were seen in crude particulate fractions.

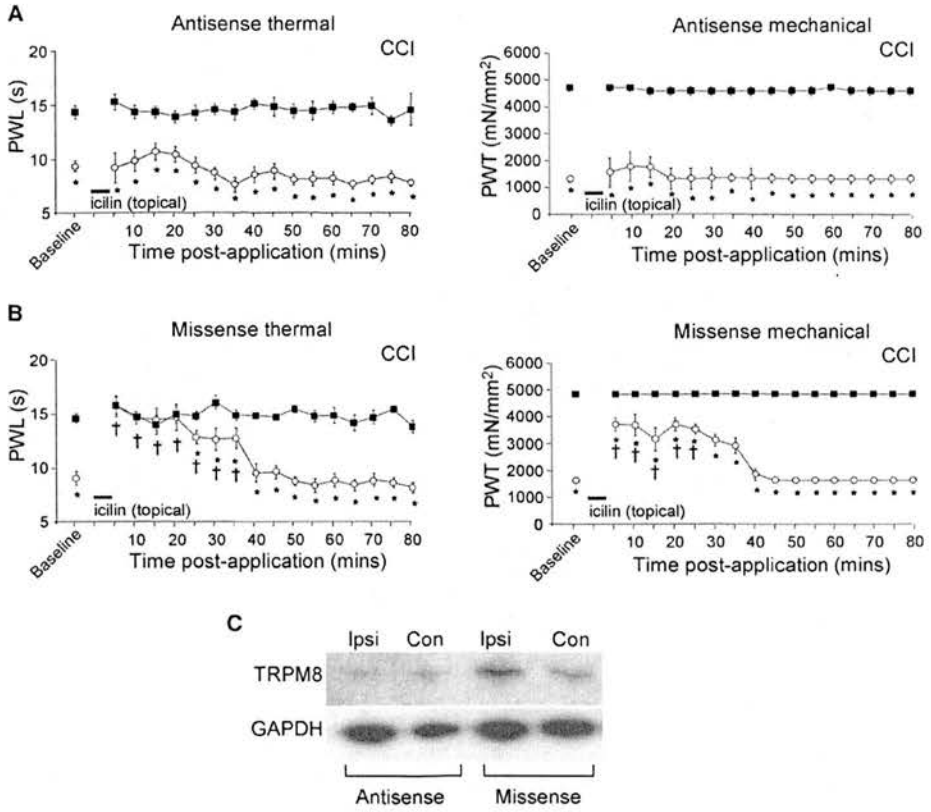

Figure 3. Specific TRPM8 Knockdown by Antisense Oligonucleotide Prevents Icilin-Induced Analgesia after CCI

(A and B) Paw withdrawal latency (PWL, s) to noxious heat and paw withdrawal threshold (PWT; mN/mm^2) to mechanical stimuli are shown ipsilateral (○) or contralateral (■) to CCI animals with antisense (n = 12) or missense (n = 10) treatments. Data show mean ± SEM.

(A) The analgesia normally elicited by topical icilin was not observed after antisense knockdown of TRPM8 receptor indicated by the persistence of significant ipsilateral-contralateral differences in PWL and PWT (* $p < 0.05$).

(B) In contrast, after missense treatment, icilin produced a significant reversal of CCI-induced ipsilateral sensitization in PWL and PWT responses in comparison to baseline values († $p < 0.05$), as in untreated CCI animals (Figure 1A).

(C) Immunoblots of DRG tissue probed for TRPM8 and GAPDH protein levels after TRPM8 antisense or missense treatment. TRPM8 expression (and the increase in expression normally seen ipsilateral to CCI, Figure 2A) was selectively reduced by antisense, but not missense, infusion, whereas GAPDH levels were unchanged.

events are likely to be important in icilin action. Icilin-responsive afferents are expected to release glutamate, so we hypothesized that inhibitory glutamate receptors in the dorsal horn might underlie icilin-induced analgesia. Group II/III mGluRs could subserve such a role because they are antinociceptive in models of inflammatory, neuropathic, and acute pain [30–32] and inhibit transmission between primary afferent and spinal cord neurons in sensitized states [48, 49]. Group II mGluRs are localized on primary afferent terminals in lamina II, particularly in small nociceptive afferents [28, 50], although some are found postsynaptically and on glia [29]. Group III mGluRs are also found presynaptically in the dorsal horn and are 45% coexpressed with either IB4 or Substance P (markers of small nociceptive neurons [51]). To assess whether activation of Group II/III mGluRs might mimic icilin reversal of neuropathic sensitization, we intrathecally injected the selective Group II

(B) shows western blots of DRG from naive or TRPM8 antisense-treated rats probed with the TRPM8 antibody used for immunohistochemistry in (C)–(F) below. Pretreatment of the antibody with the antigenic peptide or TRPM8 antisense treatment removed the single specific band at ≈128 kDa.

(C) L5 spinal cord sections taken 8 days after dorsal rhizotomy were immunostained for peripherin (green) and TRPM8 (red) and showed marked reduction of both proteins ipsilateral to rhizotomy.

(D) Immunostaining for TRPM8 (red) and the neuronal marker NeuN (green) in the spinal cord dorsal horn from CCI animals showed that TRPM8 was increased ipsilateral to CCI with no change in distribution, whereas NeuN levels were unchanged. Scale bar for (C) and (D) represents 500 μm.

(E and F) Immunohistochemical colocalization in DRG sections ipsilateral or contralateral to nerve injury and in naive animals of TRPM8 (red) with (E) NF-200 (green) or (F) peripherin (green). In naive animals, TRPM8 is mainly located in peripherin-positive C fibers, with little or no apparent expression in myelinated (NF-200) cells. Ipsilateral to nerve injury, TRPM8 expression was increased markedly in small NF-200-positive cells, whereas a lesser increase in TRPM8:peripherin coexpression was also observed. Scale bar represents 50 μm. The bar charts in (E) and (F) show the percentage coexpression (mean ± SEM) for TRPM8:NF-200 and TRPM8:peripherin, respectively; actual cell counts are shown above columns. Statistically significant increases in the percentage coexpression values were seen in both cases ipsilateral to CCI, $p < 0.05$ (*).

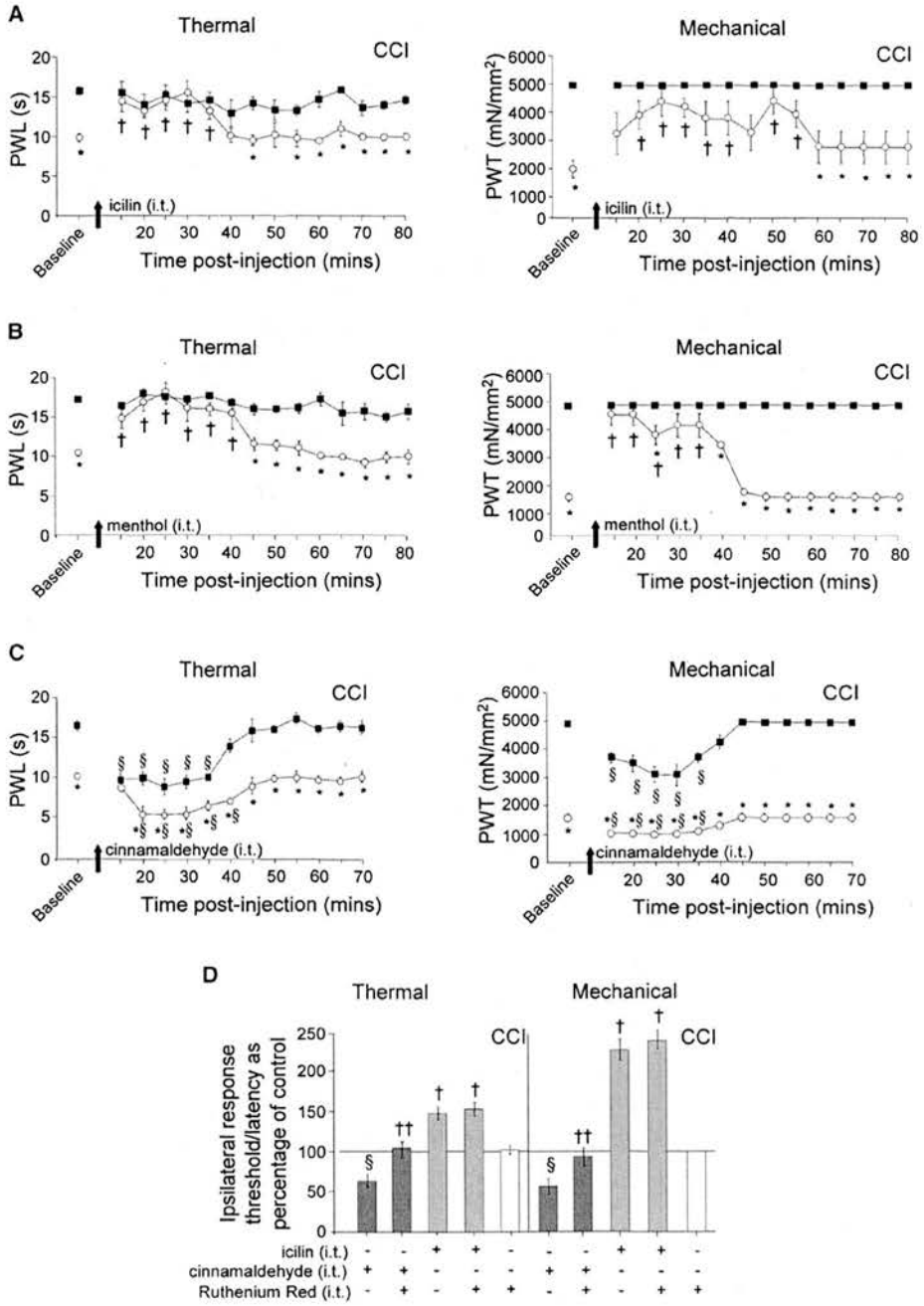

Figure 4. Central TRPM8 Activation Is Analgesic after CCI, whereas TRPA1 Activation Is Hyperalgesic

Paw withdrawal latency (PWL, s) to noxious heat and paw withdrawal threshold (PWT, mN/mm^2) to mechanical stimuli are shown ipsilateral (○) or contralateral (■) to CCI. * denotes significant ipsilateral-contralateral differences (* $p < 0.05$). Data show mean ± SEM, and each test represents n of six animals unless otherwise indicated. Rats were intrathecally injected (at arrow). The TRPM8 activators (A) icilin (10 nmol) and (B) (−)-menthol (200 nmol) both significantly reversed ipsilateral thermal and mechanical sensitization in comparison to preinjection values (†, $p < 0.05$). (C) In contrast, intrathecal application of the TRPA1 activator cinnamaldehyde (75 nmol) produced bilateral hyperalgesia and allodynia in CCI animals. § ($p < 0.05$) shows statistically significant increases in thermal and mechanical reflex responsiveness of both ipsilateral and contralateral paws.

(D) shows that Ruthenium Red (0.25 nmol) inhibits cinnamaldehyde (75 nmol)-induced hypersensitivity, but not icilin (10 nmol)-mediated analgesia, ipsilateral to CCI. Values are mean ± SEM, n = 4. Statistically significant changes in PWL/PWT values as a result of cinnamaldehyde or icilin compared to predrug baseline are shown as § and †, respectively ($p < 0.05$), and for Ruthenium Red-reversal of the effect of cinnamaldehyde (††, $p < 0.05$).

Table 2. Reversal of Sensitization by Intrathecal Icilin Administration in Different Pain Models

Pain Model	PWL (s) Difference from Control (no drug)	PWL (s) Difference from Control (+ icilin)	Mean % Reversal of Thermal Sensitization (after 15–30 min)	PWT (mN/mm^2) Difference from Control (no drug)	PWT (mN/mm^2) Difference from Control (+ icilin)	Mean % Reversal of Mechanical Sensitization (after 15–30 min)
CCI	5.9 ± 0.9[a]	0.9 ± 1.2[b]	84.7 ± 6.0	3347.4 ± 126.3 [a]	775.4 ± 467.7[b]	76.8 ± 5.3
CFA	4.7 ± 0.9[a]	1.6 ± 1.6[b]	66.0 ± 7.5	2975.6 ± 245.5[a]	1707.6 ± 149.9[b]	42.6 ± 2.8
Lysolecithin	7.8 ± 1.0[a]	2.4 ± 1.2[b]	69.2 ± 5.1	3003.8 ± 182.4[a]	1102.5 ± 422.5[b]	63.3 ± 6.4
Cinnamaldehyde (intrathecal)	5.9 ± 0.9[a]	0.5 ± 1.8[b]	91.5 ± 4.1	2022.6 ± 375.4[a]	150.3 ± 125.2[b]	92.6 ± 2.8
Cinnamaldehyde (topical)	5.0 ± 1.5[a]	0.4 ± 1.6[b]	92.0 ± 6.1	1002.0 ± 316.8[a]	0.0 ± 0.0[b]	100.0 ± 0.0

[a] Significant ipsilateral-contralateral differences in surgical pain models or, in the case of cinnamaldehyde-induced responses, differences from prior baseline, are indicated ($p < 0.05$).
[b] Significant icilin (10 nmol, intrathecal)-induced reversal of sensitization is indicated ($p < 0.05$).

or III mGluR agonists 2R, 4R-APDC or ACPT-III and AP-4, respectively. 2R, 4R-APDC (15 nmol) caused 72.1% ± 6.4% reversal of thermal and 56.0% ± 10.9% reversal of mechanical reflex sensitization ipsilateral to CCI (15–30 min postinjection) with no effect on contralateral responses. ACPT-III and AP-4 (150 nmol each) also reversed thermal sensitization (by 83.6% ± 6.3% and 60.8% ± 6.7%, respectively), as well as mechanical sensitization (65.7% ± 11.4% and 60.7% ± 8.0%), again with no effects contralaterally ($p < 0.05$ in each case). Furthermore, selective Group II and Group III mGluR antagonists LY 341495 (5 nmol, Figure 5A) and UBP 1112 (10 nmol, Figure 5B) each prevented the effect of icilin (10 nmol, Figure 4A). Similarly, the analgesia produced by intrathecal (−)-menthol (200 nmol, Figure 4B) was reversed by intrathecal LY 341495 and UBP 1112. The mean percentage reversal of sensitization over 20–30 min postinjection was 86.1% ± 8.1% for PWL and 80.6% ± 4.2% for PWT with (−)-menthol alone, 22.0% ± 6.9% for PWL and 7.1% ± 7.1% for PWT with menthol and LY 341495, and 9.2% ± 6.9% for PWL and 0.0% ± 0.0% for PWT with (−)-menthol and UBP 1112 ($n = 6$). Neither LY 341495 nor UBP 1112 had any effects alone (data not shown), suggesting that Group II/III mGluRs show little tonic activation after CCI, but are specifically utilized downstream of icilin. In contrast, intrathecal coadministration of the μ-opioid receptor antagonist naloxone (25 nmol) with icilin had no effect (Figure 5C), indicating that icilin analgesia is opioid independent. To avoid any possibility of nonspecific drug interactions, we also administered icilin (200 μM) topically, but the mGluR antagonists intrathecally. Figure 5D shows that the icilin reversal of thermal and mechanical sensitization in this case was again prevented by LY 341495 or UBP 1112. The analgesic effect of skin cooling to 16°C (Figure 1E) was also prevented by intrathecally applied LY 341495 (5 nmol) or UBP 1112 (10 nmol). The mean percentage reversal of ipsilateral CCI-induced reductions in PWT caused by cooling was 0.0% ± 0.0% in the presence of either drug ($n = 5$).

To confirm the analgesic effect of icilin at the level of single spinal cord neurons, we made in vivo extracellular recordings of large lamina I and III/IV neurons (which integrate nociceptive and nonnociceptive inputs). Topical administration of icilin (200 μM) to the peripheral receptive field area ipsilateral to CCI caused inhibition of the elevated neuronal responses to motorized rotating brush (Figure 5E). In the eight neurons out of 12 that were affected by icilin (two in lamina I and six in laminae III/IV) brush-induced responses were reduced to 37.4% ± 5.5%, $p < 0.001$). Vehicle had no effect. Contralateral neurons were unaffected (111.9% ± 8.9% of control; $n = 6$). As an example of one of the Group II/III mGluR antagonists investigated on reflexes, UBP 1112 was ionophoresed in the vicinity of recorded dorsal-horn neurons at currents of 20–60 nA. UBP 1112 reversed the effect of icilin; the brush-induced firing rate reverted to 80.2% ± 9.3% of control values (Figure 5E), but UBP 1112 had no effect alone, and nor did saline current controls.

Because some Group II/III mGluRs may be postsynaptic, we asked whether icilin could reverse the additional sensitization of behavioral-reflex responsiveness caused by intrathecally applied NMDA in CCI animals. Icilin (10 nmol) clearly attenuated the additional ipsilateral sensitization induced by 3.75 nmol of NMDA plus 0.75 nmol of its coagonist site activator, ACPC, injected intrathecally. Ipsilateral PWL values in thermal tests were 10.1 ± 0.6 s at baseline and decreased to 7.9 ± 0.3 s (15–30 min after injection of NMDA/ACPC), but increased to 14.9 ± 0.6 s in the additional presence of icilin. Contralateral values were unaltered by icilin or NMDA/ACPC. Ipsilateral PWT values in mechanical tests were 1504.2 ± 105.3 mN/mm^2 at baseline, 891.6 ± 27.3 mN/mm^2 after injection of NMDA/ACPC, and 3482.7 ± 174.3 mN/mm^2 with coinjection of icilin (mean ± SEM, $n = 6$). Contralateral values again were unaltered. A component of the central events elicited by icilin may therefore be postsynaptic, although it is important to note that functional NMDA receptors may also be present on afferent terminals [52].

Discussion

Little is known of the mechanism underlying cooling-induced analgesia, but a number of cool-sensitive ion channels, including TRPM8, have recently been identified in somatosensory afferents [13]. We now show that TRPM8 activation reverses nerve-injury-induced hypersensitivity. TRPM8 can be activated by menthol [15, 16], which is analgesic in hot-plate and acetic-acid writhing tests [11], although menthol can produce pain at very high doses [53, 54]. Here, either topical or intrathecal application of (−)-menthol produced behavioral analgesia in the CCI model of neuropathic pain, most

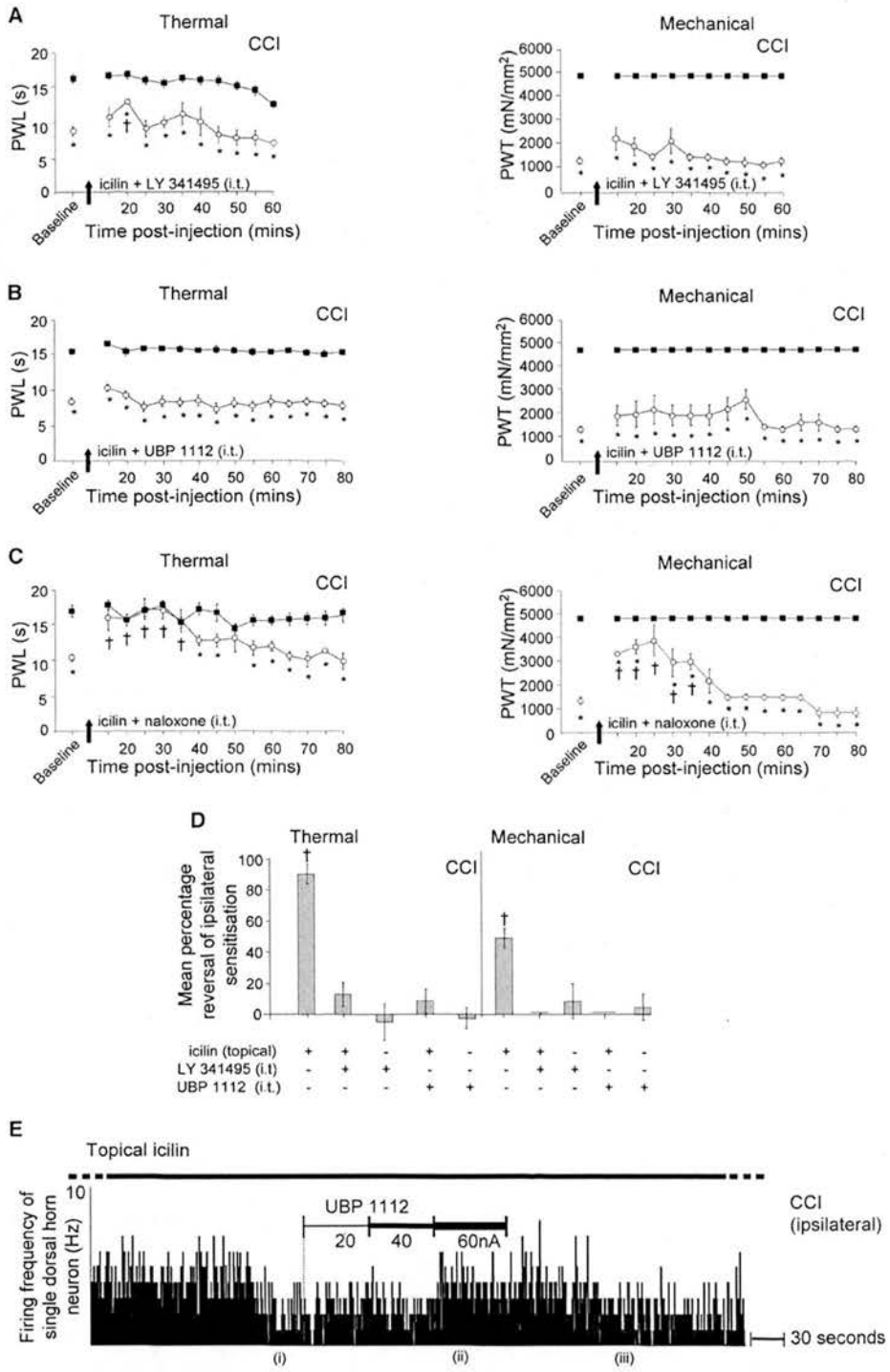

Figure 5. Icilin-Induced Analgesia after CCI Is Prevented by Group II and III mGluR Antagonists

(A–D) Paw withdrawal latency (PWL, s) to noxious heat and paw withdrawal threshold (PWT, mN/mm^2) to mechanical stimuli ipsilateral (○) or contralateral (■) to CCI. Data represent mean ± SEM, with an n of six animals in each case. Rats were intrathecally injected (at arrow). Icilin (10 nmol) was coadministered with (A) the Group II mGluR antagonist LY 341495 (5 nmol), (B) the Group III mGluR antagonist UBP 1112 (10 nmol), or (C) the opioid receptor antagonist, naloxone (25 nmol).

(A and B) When either LY 341495 or UBP 1112 was coadministered with icilin, it abolished the analgesic effect of icilin as indicated by the persistence of sensitized (*, $p < 0.05$) PWT/PWL responses ipsilateral to nerve injury.

(C) Naloxone coadministration with icilin did not prevent the icilin-induced analgesia seen in PWT and PWL responses ipsilateral to nerve injury (†, $p < 0.05$). * indicates significant ipsilateral-contralateral differences, and † indicates significant ipsilateral analgesic effect of drug ($p < 0.05$).

likely by activation of TRPM8. Similar effects were seen with another TRPM8 activator, icilin [15]. As with menthol, very high doses of icilin were found to cause a generalized increase in sensitization, affecting CCI animals bilaterally and naive animals in a similar fashion (Table 1). Importantly, analgesic effects of icilin were seen at 200-fold-lower concentrations than those causing nonspecific sensory changes. Specific involvement of TRPM8 in the reversal of neuropathic sensitization was confirmed by the abrogation of icilin analgesia after intrathecal infusion of a TRPM8 antisense oligonucleotide to knock down TRPM8 expression. Furthermore, the analgesic profile was mimicked by cutaneous cooling to 20°C–16°C, a range activating the TRPM8 channel [14]. Icilin and menthol were applied cutaneously in solution at 30°C, so any possible drug effects on skin temperature were avoided. TRPM8 antisense had no effect alone on CCI-induced sensitized responses to noxious heat, mechanical stimuli, or intense cold (Figure 3), similar to observations made in an alternative neuropathic pain model [27]. A role for the TRPA1 channel in analgesia seems unlikely because selective TRPA1 activators, cinnamaldehyde, allicin, and diallyl disulphide, caused contrasting sensitization/hyperalgesia not only after CCI, but also in naive animals. The analgesic effects of icilin were only seen in the sensitized pain state, but were not restricted to nerve injury, because sensitization due to peripheral inflammation, afferent demyelination, and TRPA1 activation was also reduced. The significant behavioral and electrophysiological effects of topically applied icilin demonstrate that icilin can cross the skin sufficiently to excite peripheral afferents, and point to the likely clinical utility of this or related drugs.

The precise identity of the TRPM8-containing cool-responsive afferents is not clear. Subpopulations of Aδ fibers and C fibers are responsive to different ranges of cool temperatures: ~15°C–30°C and <15°C [55, 38]. Innocuous cooling (15°C–30°C) activates a subpopulation of Aδ fibers and C fibers in primates, but almost solely unmyelinated fibers in rodents [55]. In contrast, intense noxious cold is signaled by unmyelinated polymodal nociceptors, which also respond to heat and mechanical stimuli [38]. The TRPM8 activator menthol activates cool-sensitive fibers and sensitizes stimulus-induced responses in the range 20°C–30°C [56, 57]. Studies of TRPM8 in vitro identify this channel as a likely transducer of moderate cool temperatures [20]. In DRG and trigeminal cultures, responses to menthol, cooling (15°C–30°C), and TRPM8 mRNA expression all correlate closely [18–20]. TRPM8 is expressed in 5%–20% of DRG cell bodies that are small and presumed Aδ fibers or C fibers [15, 16], but not in large myelinated fibers. We observed that TRPM8 immunoreactivity is normally associated with a subpopulation of peripherin-positive C fibers, but only minimally with NF-200-positive afferents, whereas high-sensitivity cRNA hybridization suggests the presence of some TRPM8 mRNA in up to 19% of NF-200-positive afferents [58]. The capsaicin- and heat-sensitive TRPV1 channel, which contributes to thermal nociception and inflammatory sensitization [12], is found both in peptidergic afferents (~85%) and in nonpeptidergic (isolectin-B4, IB4-positive) cells in the rat [59]. TRPM8 is not categorically associated with either peptidergic or IB4-positive afferents [15] but is often present in those containing the NGF receptor, Trk A [58]. Different groups have reported coexpression of mRNAs for TRPM8 and TRPV1 or menthol/capsaicin responsiveness of DRG at 29%–50% [15, 20, 60] or close to zero [14, 16, 58]. Overall, it seems likely that TRPM8 is normally expressed in a distinct population of cool-responsive afferents and possibly also to an extent in some nociceptors. Our findings further identified increased expression in peripherin-positive C fibers, but induction in small NF-200-positive presumed Aδ fibers [35], suggesting that plasticity in TRPM8 expression may participate in icilin analgesia in neuropathic pain. No changes in DRG expression of TRPM8 were reported in an alternative neuropathic pain model (ligation of L5 spinal nerve or indeed after CFA) [26, 27], suggesting that specific aspects of the particular model, such as the coexistence of injured and uninjured afferents in DRG after CCI, may be important in TRPM8 upregulation.

TRPA1, which has also been proposed as a cool receptor [14], appears to play an entirely different role, eliciting reflex pain behaviors in naive animals, as well as increasing thermal and mechanical responsiveness in the neuropathic state. This may correspond to clinical observations after nerve injury in which moderately cool stimuli are perceived as painful [1]. TRPA1 is present mainly in small cells in sensory ganglia [14, 21] and may increase ipsilateral to nerve injury and inflammation [26, 27]. Nerve-injury- and inflammation-induced hyperalgesia to noxious cold (5°C) is reported to be decreased by antisense knockdown of TRPA1 [26, 27]. Correspondingly, mutant mice homozygous for targeted disruption of the TRPA1 gene show reduced reflex withdrawal responses to selective TRPA1 activators and reduced sensitization of noxious heat and innocuous mechanical responses caused by these agents [24]. However, the role of TRPA1 in noxious cold responses is disputed, with results from different lines of TRPA1$^{-/-}$ animals showing either attenuated or unaltered coldplate withdrawal responses [24, 25]. Thus the precise role of TRPA1 in cold sensation remains unclear, but here as in other studies TRPA1 clearly acts in a pronociceptive manner [21, 22, 46, 47]. Although icilin may interact with low

(D) The analgesic effects of topically applied icilin (200 μM at 30°C) were also reversed by intrathecal injection of Group II/III mGluR antagonists. The figure shows mean % reversal of the ipsilateral/contralateral difference in either PWL or PWT measured over 15–30 min after 5 min topical application of icilin, with or without concurrent intrathecal injection of LY 341495 (5 nmol) or UBP 1112 (10 nmol), or after mGluR antagonists alone.

(E) Typical extracellular recording of a single dorsal-horn neuron ipsilateral to CCI, responding to continuous motorized brushing of the cutaneous receptive field on the hind paw and the effects of icilin (200 μM at 30°C) topically applied to an adjacent area of the receptive field. Similar results were observed in 8 out of 12 neurons with examples in both laminae III/IV and lamina I. Neuronal firing is displayed as action potentials per second (Hz) plotted against time. (E_i) Brush-evoked firing in neurons ipsilateral to nerve injury was consistently inhibited by topically applied icilin; (E_{ii}) this effect was reversed by ionophoresis of UBP 1112 at 20–60 nA; (E_{iii}) recovery was observed after removal of the UBP 1112 ejection current. Neurons contralateral to nerve injury were unaffected by icilin, and topical vehicle had no effect. In addition, ionophoresis of UBP 1112 alone or saline current controls showed no discernable effect.

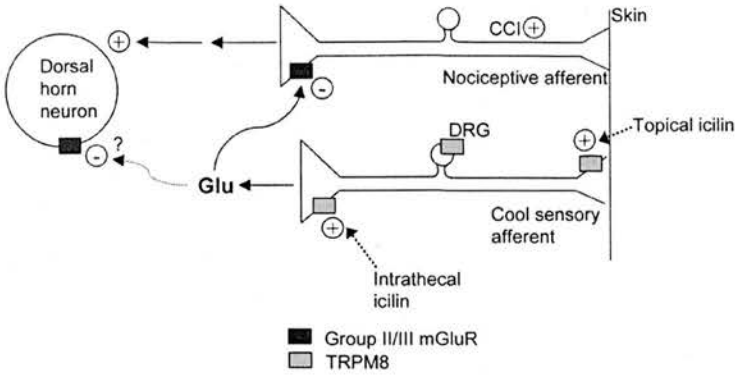

Figure 6. Schematic Representation of a Possible Mechanistic Basis for TRPM8-Mediated Analgesia after CCI

In this simplified hypothetical model, activation of TRPM8 in a subpopulation of afferents by icilin, menthol, or moderate cooling leads to central synaptic release of glutamate (Glu), which then acts through inhibitory Group II/III mGluR receptors located either presynaptically on injury-activated nociceptive afferents or perhaps also postsynaptically on dorsal-horn neurons, thereby attenuating neuropathic sensitization.

potency at the TRPA1 channel [14], the analgesic profile of TRPM8 activators here is entirely different from the pronociceptive profile of TRPA1 activators. The extent of TRPM8/TRPA1 coexpression in afferents is reported to be minimal [14, 58].

The analgesia induced by icilin and menthol and by skin cooling to 16°C was shown to be centrally mediated and dependent on Group II/III mGluRs. The lack of effect of naloxone suggests independence from classical opioid analgesia. Furthermore, at the doses used, mGluR Group II/III antagonists selectively reversed icilin and menthol analgesia in sensitized responses, without any effects alone. Group II/III mGluRs are known to inhibit nociceptive responses [30–32, 48, 49], and we showed that Group II/III mGluR agonists selectively inhibit sensitized responses in neuropathic pain. Both Group II and III mGluR subtypes are expressed in primary afferents, especially IB4-positive cells [28, 50, 51]. Activation of Group II/III mGluRs can inhibit afferent-evoked potentials in the dorsal horn [48], because Group II mGluRs are both pre- and postsynaptic at primary afferent synapses [29], whereas Group III mGluRs are largely presynaptic [51]. Menthol is reported to increase mEPSC frequency at some synapses between DRG and dorsal-horn neurons in culture and in slices [44, 45], presumably corresponding to the activation of TRPM8-containing afferents (Figure 1D) and increased release of glutamate. Figure 6 shows a schematic outline of a model in which glutamate released from TRPM8-expressing afferents could mediate icilin-induced analgesia by acting on Group II/III mGluRs (located presynaptically on nociceptive afferents and possibly also postsynaptically) to result in attenuation of pain-related sensitization (Figure 5E) and behavioral analgesia (Figure 5D).

Conclusions

In summary, these novel findings show that both peripheral and central activation of TRPM8 can produce an analgesic effect that specifically reverses the sensitization of behavioral reflexes elicited by peripheral nerve injury. This effect is produced by very low concentrations of topically applied TRPM8 activators, pointing to the likelihood of its ready utility in a clinical context. Other sensitized pain states, in addition to that induced by nerve injury, are similarly sensitive to reversal by TRPM8 activation, emphasizing the likely value of TRPM8 activators and downstream central mediators of TRPM8 action, such as Group II/III mGluRs, as targets for the development of novel analgesics.

Experimental Procedures

Animals

All experiments were conducted in accordance with the UK Animals (Scientific Procedures) Act, 1986, and guidelines of the University of Edinburgh. Adult male Wistar rats (Harlan, United Kingdom) weighing 120–250 g were used for all experiments.

Neuropathic and Inflammatory Pain Models

Pain models were generated under halothane anesthesia (Zeneca, Cheshire, United Kingdom). For the chronic constriction injury (CCI) model of neuropathic pain, four ligatures were tied loosely to constrict the sciatic nerve at midthigh level (as described previously [61]). An inflammatory pain model was generated by injecting 100 μl of Complete Freund's Adjuvant (CFA, Sigma-Aldrich) into the ventral surface of the right hind paw [61]. A model of peripheral demyelination-induced pain was produced by focal application of lysolecithin to the sciatic nerve [43]. Peak behavioral sensitization was observed postsurgically between days 10 and 16 for CCI, 1 and 3 for CFA, and 7 and 14 for lysolecithin, when pharmacological and electrophysiological experiments and tissue removal were conducted.

Behavioral Testing

Thermal sensitivity was assessed by measuring paw withdrawal latency (PWL, s) in response to a noxious thermal stimulus (Hargreaves' thermal stimulator, Linton Instrumentation, Diss, United Kingdom) directed to the hind paw midplantar glabrous surface. Mechanical sensitivity was recorded as the paw withdrawal threshold (PWT, mN/mm^2) to calibrated von Frey filaments (Stoelting, Illinois), as previously described [61]. Sensitivity to noxious cold was assessed by placing animals in a water bath with an aluminium floor containing 1-cm-deep 4°C water and counting the time the paw was held suspended over a 20 s period.

Intrathecal Application of Drugs

The following drugs were applied intrathecally in a 50 μl volume of saline-based vehicle at 37°C: icilin (2.5–200 μM in saline with 0.2% dimethylformamide, DMF); LY 341495 (100 μM in saline); UBP 1112 (200 μM in saline); 2R, 4R-APDC [(2R, 4R)-4-aminopyrrolidine-2,4-dicarboxylate, 300 μM in saline]; ACPT-III [(1R,3R,4S)-1-aminocyclopentane-1,3,4-tricarboxylic acid, 3 mM in saline]; AP-4 [(L-(1)-2-amino-4-phophonobutyric acid, 3 mM in saline); naloxone (0.5 mM in saline); NMDA (75 μM in saline) and ACPC (1-aminocyclopropanecarboxylic acid, 15 μM in saline) (Tocris Cookson, Bristol, United Kingdom); (−)-menthol [1R, 2S, 5R-(−)-menthol, 4 mM in saline]; cinnamaldehyde (1.5 mM in saline); Ruthenium Red (5 μM in saline) (Sigma-Aldrich, United Kingdom); allicin (0.5 mM in saline with 0.5% DMF); and diallyl disulphide (DADS, 1 mM in saline with 0.5% DMF) (LKT Laboratories, St. Paul, Minnesota). Drugs were injected into the L5–6 intrathecal space under brief halothane anesthesia, with a 25-gauge needle microsyringe (BD Biosciences, Oxford), as described previously [61] in animals that were at peak levels of

behavioral sensitization. Behavioral-reflex testing commenced 15 min after injection to allow recovery from anesthesia [43, 62, 63] and continued every 5 min thereafter until readings returned to baseline levels (n = 6 in each case). We, and others [43, 62, 63], find complete recovery from anesthetic by 15 min. All appropriate controls were carried out to eliminate the possibility of effects due to vehicle or to injection procedure.

The TRPM8 channel activators icilin and (−)-menthol were tested in CCI and naive animals. The effects of icilin were additionally assessed in animals with CFA-induced inflammation or with lysolecithin-induced demyelination. Icilin was also coadministered with the μ-opioid receptor antagonist naloxone, the Group II metabotropic glutamate receptor (mGluR) antagonist LY 341495, or the Group III mGluR antagonist UBP 1112. The effects of these antagonists alone, as well as effects of the Group II mGluR agonist 2R, 4R-APDC and the Group III mGluR agonists ACPT-III and AP-4, were assessed in CCI animals. The TRPA1 channel activator cinnamaldehyde, alone and with icilin, was tested in CCI animals and in naive animals. Further TRPA1 channel activators, allicin and diallyl disulphide, were assessed in naive animals. Both icilin and cinnamaldehyde effects in CCI animals were also investigated in the additional presence of Ruthenium Red.

Topical Application of Drugs

Icilin was applied at concentrations of 2.5–500 μM (in water with 0.2% DMF), by placing CCI or naive rats unrestrained for 5 min in a 1-cm-deep water bath (sufficient to cover paws), which was thermostatically controlled to a temperature of 30°C, or by very lightly anesthetizing rats and immersing hind paws in small tubes containing 5 ml icilin (500 μM–5 mM, with a vehicle of 45% dimethylformamide in 0.2% aqueous Tween 80) for 5 min, followed by sensory testing for 60–80 min. The effects of (−)-menthol and its stereoisomers isomenthol (1S, 2R, 5R-menthol,) and (+)-menthol [1S, 2R, 5S (+)-menthol] (4–16 mM in 80% ethanol) were also assessed. Relevant vehicles were always assessed in similar experiments. As a contrast, the effect of cinnamaldehyde (1.5 mM in water) and the effect of cinnamaldehyde with additional icilin (80 μM in water with 0.2% DMF) were assessed in naive animals. The effects of icilin (80 μM) were further assessed in CCI animals that had undergone antisense and missense treatment for knockdown of TRPM8 or immediately after intrathecal injection of either LY 341495 or UBP 1112. Actual skin temperatures were measured by a subcutaneous thermistor probe and were found to equilibrate to around 0.5°C above bath temperature. The effects of brief paw immersion at different temperatures (10°C–22°C for 5 min) on CCI rats were assessed by mechanical testing. The effects of intrathecal LY 341495 or UBP 1112 on the reversal of ipsilateral sensitization in CCI after a 16°C cool challenge for 5 min were measured. Reflex testing commenced 5 min after challenge, unless animals had been anesthetized, in which case 15 min was allowed for recovery. Six replicate animals were tested in all pharmacological experiments.

Dorsal Rhizotomy

To establish whether TRPM8 expression in the spinal cord was predominantly pre- or postsynaptic, we performed a unilateral L2–6 dorsal rhizotomy under anesthesia, following laminectomy to expose the dorsal roots. Eight days later, tissue was removed and processed for immunohistochemistry.

Western Blots

Experiments were performed by standard procedures as previously described [61]; for more details see the Supplemental Data available online.

Immunohistochemistry

Experiments were performed as previously described [43]; for more details, see Supplemental Data.

Antisense Knockdown of TRPM8

Antisense and missense oligonucleotides were 22 mers with phosphorothioate bonds at the last two positions at the 5′ and 3′ ends (MWG Biotech, Ebersberg, Germany). Antisense extended from base −10 to base +12 relative to the start of the open reading frame for the rat TRPM8 gene: 5′ C*T*CGAAGGACATCTTGCCGT*G*G 3′, where * represents phosphorothioate linkages. Missense was designed with four inversions of C/G or A/T as appropriate at residues 3, 11, 14, and 22, preserving overall G/C content. BLAST searches of both oligonucleotides indicated no significant complementarity to any known gene sequence. Fourteen day or seven day osmotic minipumps (for CCI experiments or naive electrophysiology experiments, respectively—Alzet Minipump, models 2002, 2001; Charles River, United Kingdom) containing oligonucleotides (1 μg/μl in sterile saline) were connected to canulae inserted under the dura of the spinal cord to level L5–6 and produced a predicted infusion rate of 0.5 μl/hr. CCI surgery was performed at the same time as minipump implantation. Sensory tests were carried out to assess the time course of behavioral sensitization in animals that had also undergone a CCI injury. Icilin was applied topically rather than intrathecally so as to prevent any interference with the infusion canula. Peripheral nerve recordings and tissue harvesting were carried out after an interval of 4–5 days to allow time for protein knockdown.

Electrophysiology

Peripheral

Recordings of saphenous (sensory) nerve were made in naive animals (n = 7) to assess the effects of topical icilin on primary afferents. In addition, recordings were carried out on animals that had undergone TRPM8 antisense or missense treatment beginning 4–5 days previously. Rats were anesthetized (with 0.6 ml 25% urethane/100 g, intraperitoneal), and the saphenous nerve was exposed and dissected from its associated vein and artery. Further dissection under liquid paraffin enabled identification of afferent preparations comprising a small number of fibers. The conduction velocity of single identified afferent fibers was determined by using bipolar electrodes and the peripheral stimulus technique [64]. After isolation of preparations, icilin (200 μM in water with 0.2% DMF), (−)-menthol (4 mM in 25% ethanol), resiniferatoxin (1 mM in ethanol), or vehicle alone was applied to the hind limb receptive fields, and neuronal responses were recorded with the Chart program (version 3.6).

Central

Recordings of spinal dorsal-horn neurons were made in CCI animals, as described previously [61]. After halothane induction, the jugular vein and trachea were canulated and intravenous anesthetic was delivered: α-chloralose (0.6 mg/kg, Fisher) and urethane (1.2 mg/kg, Sigma), with small supplementary doses of α-chloralose as required throughout the experiment. Core body temperature was maintained at 37°C–38°C by means of a thermostatically controlled heated blanket. The animal was placed in a stereotaxic frame, and the thoracolumbar spinal column was supported by three pairs of swan-necked clamps. A laminectomy was performed at L2–L5, and agar (2% in saline at 37°C) was delivered over exposed cord to increase mechanical stability. Above the recording region, the agar and spinal cord dura were removed, and liquid paraffin was poured into the pool. Extracellular recordings were made from single multireceptive neurons in laminae I–IV through the center barrel of a seven-barrelled glass microelectrode filled with 4 M NaCl (tip-diameter 4–5 μm, DC resistance 5–8 MΩ). The receptive fields of hair-follicle innervated neurons on the distal hind limb were identified by an innocuous brush stimulus [61]. Icilin (200 μM in water with 0.2% DMF) was applied peripherally to the receptive field of individual recorded neurons, and the effect on neuronal response to a rotating brush was recorded and analyzed with Spike2 program (Version 3.2, CED). The Group III mGluR antagonist UBP 1112 (20 mM in water), pH 8.5, and control 1 M NaCl, pH 8.5, were ionophoresed from the side barrels of the electrode by using currents of between 20 nA and 80 nA (Neurophore BH2 ionophoresis system, Medical Systems, Great Neck, New York) to measure effects on neuronal response to icilin.

Statistics

All data were analyzed for statistical significance by using Sigmastat software (version 2.03) with p values < 0.05 being considered significant. Differences in thermal sensitivity between the paw ipsilateral to nerve injury and the contralateral paw were assessed with Student's t test. Any effect of drug treatment was analyzed by one-way repeated-measures ANOVA followed by Dunnett's post-hoc multiple-comparisons test. The equivalent nonparametric tests for mechanical sensitivity were Wilcoxon rank test for

ipsilateral:contralateral differences and Friedman repeated-measures ANOVA followed by Dunn's test for changes from predrug control values. Western blot-densitometric values were compared by using the Wilcoxon test, immunohistochemistry cell counts were analyzed by one-way ANOVA, and electrophysiological spike frequencies were analyzed by one-way ANOVA on ranks.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and are available with this article online at: http://www.current-biology.com/cgi/content/full/16/16/1591/DC1/.

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Chapter 27

The Ubiquitin Proteasome System in Pain Transmission and Neuropathic Pain

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ABSTRACT

Chronic pain states involve long-term biochemical and anatomical changes including plasticity at the first synapse in the spinal cord, which is crucial to the development of hyperalgesia (increased sensitivity to noxious stimuli) and allodynia (perception of innocuous stimuli as painful). Spinal dorsal horn neurons become hyperexcitable in the process of "central sensitisation", which shows partial similarity to other forms of synaptic plasticity, such as hippocampal long-term potentiation (LTP). Both processes involve pre- and post-synaptic changes, and rely on the NMDA receptor and associated proteins. The ubiquitin-proteasome system (UPS) has been implicated in central sensitisation and the development of neuropathic pain. In an animal model of neuropathic pain, proteasome inhibitors have been shown to rapidly attenuate behavioural hyperalgesia and allodynia, inhibited firing of dorsal horn neurons evoked by noxious and innocuous stimuli in neuropathic animals, or by mustard oil in normal animals. Expression of the enzyme UCH-L1 (Ubiquitin C-terminal hydrolase) was further increased in the spinal cord dorsal horn ipsilateral to neuropathy, supporting a central role for the UPS in neuropathic pain. Studies of other CNS areas have emphasised the importance of the UPS in regulation of synapse structure and neurotransmitter release and its role of such changes in plasticity. Postsynaptically, the UPS mediates changes in composition of the postsynaptic density (PSD) since activity-dependent ubiquitination regulates PSD composition and several key scaffolding molecules which are involved in pain sensitisation, undergo activity-dependent ubiquitination. PSD-95 in particular plays a key role in neuropathic pain. PSD-95 links to the NMDA receptor which is essential for central sensitisation and may regulate AMPA receptor synaptic insertion. NMDA receptor activation causes PSD-95 ubiquitination and degradation and blockade of this process prevents NMDA receptor induced AMPA-receptor recycling and long-term depression. Since alteration in levels of surface glutamate receptor expression is a key means by

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which synaptic strength is altered, these observations further support the idea of acute regulation of synapse function by the ubiquitin-proteasome pathway. In addition, proteasome inhibitors reduce NMDA receptor-dependent activation of the CREB and ERK/MAPK signalling cascades, indicating a further mechanism by which the UPS may influence long-term plasticity during chronic pain. The UPS is also involved in synapse development and in morphological changes in dendritic spines. Thus, a wide array of changes in protein:protein interactions, signalling events and cytostructural changes depend on UPS function during the synaptic plasticity that underlies chronic pain states. These processes may represent promising targets for the development of novel analgesic strategies.

Key words: Pain, Neuropathy, Ubiquitin, Proteasome, Neuronal plasticity, Posterior horn, Postsynaptic density.

Abbreviations:

CCI; chronic constriction injury model
CFA; Complete Freund's adjuvant
CNS; central nervous system
COX-2; cyclooxygenase-2
CREB; cyclic AMP response element binding protein
DRG; dorsal root ganglia
E1; ubiquitin-activating enzyme
E2; ubiquitin conjugating enzyme
E3; ubiquitin ligases
ERK; extracellular signal-regulated protein kinase
GluR; glutamate receptor (AMPA receptor subtype)
GRIP; glutamate receptor -interacting protein
IKK; I_KB kinase
IL; interleukin
LI/II; laminae I and II in superficial dorsal horn of spinal cord
LTP; long-term potentiation
MAGUK; membrane associated guanylate kinase
MAP; mitogen activated protein
mGluR; metabotropic glutamate receptor
NF-L; neurofilament-light
NR; NMDA receptor
NSAIDs; Non-steroidal anti-inflammatory drugs
NSF; N-ethylmaleimide-sensitive fusion protein
PICK1; protein interacting with C kinase 1
PKA; protein kinase A
PWL; paw withdrawal latency
PWT; paw withdrawal threshold
PSD; postsynaptic density
RVM; rostroventral medulla
Ser; serine reside
Siah1A; Seven in absentia homolog 1A

SPAR; spine-associated Rap GTPase activating protein
SPET; suspended paw elevation time
TNF-α; tumour necrosis factor-α
UCH-L1 (Ubiquitin C-terminal hydrolase)
UPS; ubiquitin proteasome system

INTRODUCTION

Normal, physiological pain serves a useful purpose, alerting us to damaging stimuli and triggering withdrawal reflexes, or forcing a state of rest to promote recovery from injury. On the contrary, chronic pain, which persists long after the original cause has resolved, serves no beneficial purpose and presents a major clinical problem. Neuropathic pain is a form of chronic pain which is caused by damage to the nervous system. It can occur as a result of trauma, diabetes and demyelinating diseases, viral infections (such as in postherpetic neuralgia), certain chemotherapeutic agents (all of which damage peripheral nerves) and also from central nervous system damage. Treatment options are limited [1]; conventional first-line treatments such as NSAIDs (Non-steroidal anti-inflammatory drugs) and opioids are ineffective [2]. Current prescribed medications are anti-convulsant drugs (e.g. gabapentin), sodium channel blockers and tricyclic antidepressants, all of which show variable efficacy and cause deleterious side effects. Under normal circumstances, a sensation of pain is evoked when noxious stimuli to the periphery evoke action potentials in the terminals of nociceptive-specific primary afferent neurons. These primary afferents terminate in the dorsal horn of the spinal cord, synapsing onto central spinal neurons, which form several pathways connecting to the thalamus and cortex. The synapse in the dorsal horn between primary afferent and second order neurons plays a key role in pain transmission and processing. Plasticity at this synapse is a major means by which behavioural sensitisation can develop. This sensitisation is reflected in hyperalgesia — heightened sensitivity to noxious stimuli, allodynia — perception of innocuous stimuli as noxious, and spontaneous pain. Various animal models have been developed in an effort to reproduce the sensory disorders accompanying human peripheral neuropathies. Partial nerve injury models mimic the most common type of injury seen in humans. Such models are highly reproducible and result in the hallmark behavioural signs of neuropathic pain; hyperalgesia and allodynia.

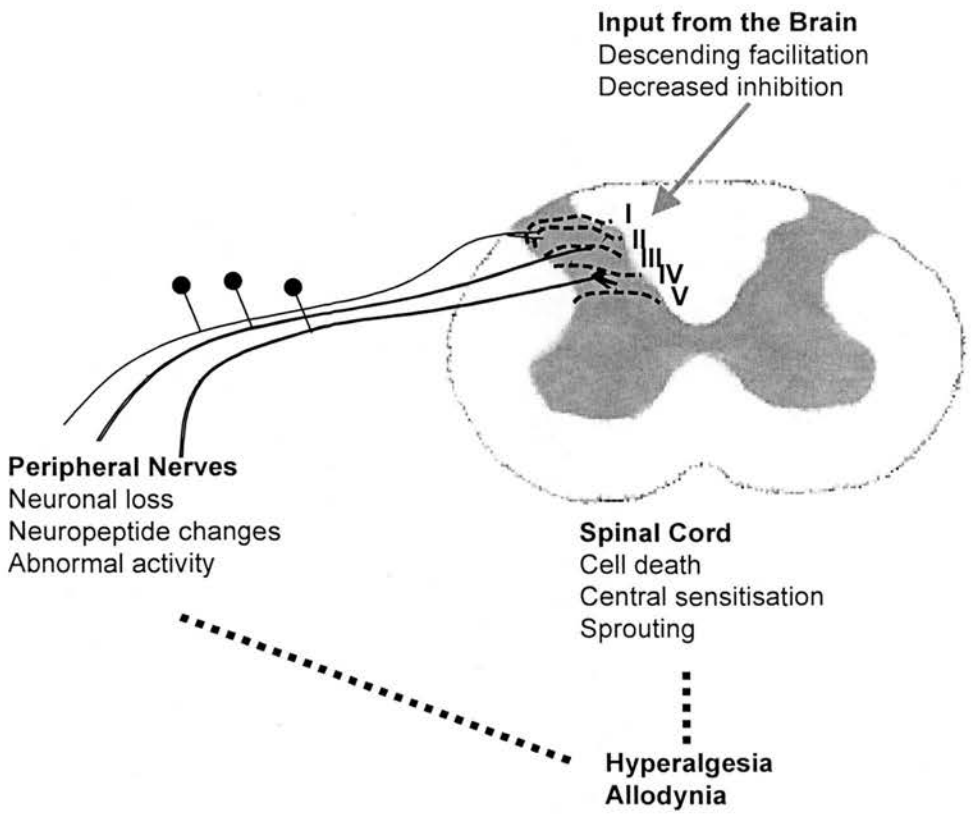

Figure 1: Peripheral and central mechanisms of neuropathic pain

Changes at the level of peripheral afferents, the dorsal horn of the spinal cord, and descending input from brain areas. All lead to the development of hyperalgesia (heightened sensitivity to noxious stimuli) and allodynia (perception of innocuous stimuli as painful) in neuropathic pain.

Sensitisation as a result of plasticity in pain pathways occurs normally following injury, but is short-term and reverses as the injury heals. However, in neuropathic pain, continuing modification of the pain pathways, particularly at the early synapses in the dorsal horn, results in long-term sensitisation, which far outlasts the injury. Damage to the peripheral nerves causes some axons to degenerate, and remaining neurons display altered properties including spontaneous and ectopic firing. Phenotypic changes occur in damaged and neighbouring afferents with alterations in expression of ion channels, receptors, and levels of neurotransmitters, and *de novo* expression of certain proteins. In postsynaptic neurons, levels of receptors and cellular proteins are also altered. These changes result in central sensitisation, a form of synaptic plasticity with some analogies to hippocampal long-term potentiation (LTP) and to other forms of central nervous system plasticity resulting in a facilitation of neurotransmission due to repetitive conditioning stimuli (see ***Chapter 14-17***; ***Figure 1***). Activity-dependent sensitisation has been reported in rodent, cat and primate dorsal horn neurons [3, 4]. A central sensitisation-like phenomenon can also be generated in

other supraspinal pain processing areas: the rostroventral medulla (RVM), anterior cingulate cortex, and amygdala [5, 6, 7].

LTP and central sensitisation are both dependent on excitatory glutamatergic transmission involving AMPA and NMDA subtypes of ionotropic glutamate receptor [8, 9, 10, 11, 12]. AMPA receptors are composed of four subunits (GluR1-4), while NR1, NR2 (NR2A-D) and NR3 (NR3A/B) subunits all constitute the NMDA receptor family. All these subunits are differentially localised in the spinal cord with GluR1 and GluR2 subunits of AMPA receptor and NR1 and NR2B subunits of NMDA receptor being most concentrated in the major nociceptive processing area in the superficial dorsal horn (see later). Under basal conditions, acute nociceptive transmission is mediated by AMPA receptors, while NMDA receptors are inactive, due to blockade by Mg^{2+} ions in the NMDA channel pore. Under conditions of nerve injury, high-frequency stimulation produces a cumulative membrane depolarisation which leads to removal of the voltage-dependent Mg^{2+} ion block in the channel pore, allowing entry of calcium ions and resultant calcium-dependent intracellular signalling. Metabotropic glutamate receptors (mGluR1-7) have also been implicated in synaptic plasticity in hippocampus and cerebellum as well as spinal neuronal sensitisation associated with chronic pain.

THE UBIQUITIN-PROTEASOME SYSTEM (UPS)

A major intracellular pathway that is now known to act as an important regulator of such synaptic function is the ubiquitin-proteasome system [13, 14, 15, 16]. The UPS is a highly conserved multi-enzyme mechanism that targets cytosolic proteins for degradation [17]. A sequential series of reactions is catalysed by ubiquitin-activating enzyme (E1), followed by ubiquitin conjugating enzymes (E2) and a number of ubiquitin ligases (E3), resulting in ubiquitin binding to lysine residues in the target protein [18]. Activity-dependent ubiquitination and degradation by the proteasome proteolytic complex is a mechanism by which synaptic proteins and thus synaptic strength may be regulated. Levels of ubiquitin-conjugated proteins are strongly linked to synaptic activity level, and this is particularly pronounced for postsynaptic density (PSD) proteins [19]. This pathway is involved in the regulation of synaptic plasticity in the hippocampus, whereby the proteasome inhibitors MG-132 and lactacystin can block long-term depression, a further form of lasting alteration in synaptic function [16].

LOCALISATION OF THE UPS NEAR TO SYNAPSES:

The UPS has been localised intracellularly in the vicinity of the synapse, making it a plausible regulator of synaptic function. Confocal microscopy demonstrates that ubiquitin and the proteasome are abundant in dendrites of cultured hippocampal neurons, in or near putative spines [15]. Ubiquitin conjugation has been demonstrated in synaptosomal and PSD preparations, indicating that ubiquitination can occur in or near synapses [19]. It is possible that dynamic recruitment of UPS components to synaptic sites could act as a regulator of function [15]. The UPS is directly involved in affecting growth of dendritic spines by a mechanism involving degradation of the protein SPAR (spine-associated Rap GTPase activating protein) which is a postsynaptic scaffolding molecule that binds both filamentous

actin and the MAGUK (membrane-associated guanylate kinase family adapter protein prominent in postsynaptic densities, PSD-95. Degradation of SPAR by the UPS is activity-dependent, and results in depletion of both SPAR and PSD-95 from spines, leading to dissipation of spines [20].

THE UPS AND NEUROPATHIC PAIN

The UPS is specifically implicated in the sensitisation that occurs in the spinal cord in chronic pain [21]. Extracellular recordings of multireceptive dorsal horn neurons show heightened neuronal activity following the topical application of the chemical algogen mustard oil (allyl isothiocyanate), a selective activator of nociceptive C-fibers [22]. Ionophoresis of the ubiquitin-proteasome inhibitors lactacystin or MG-132 *in vivo* partially inhibits the mustard oil-induced increase in firing but had no effects on responses to an innocuous brush stimulus [21]. This indicates that the UPS is preferentially involved in the processing of noxious (mustard oil) but not innocuous (brush) sensory stimuli in the spinal cord under normal conditions (***Table 1***).

Table 1. Summary of effects of proteasome inhibitors on sensory stimulation-induced dorsal horn neuronal activity

	Normal			Nerve injury		
Stimulus type	Innocuous	Noxious		Innocuous	Noxious	
	Brush	Mustard Oil	Cold	Brush	Mustard Oil	Cold
Lactacystin	-	↓	n/a	↓	↓	↓
MG-132	-	↓	n/a	↓	↓	↓

Summary of the effects of the ionophoretic application of proteasome inhibitors lactacystin and MG-132 on dorsal horn neuron responses to innocuous (brush) or noxious (mustard oil or cold) sensory stimulation. In naïve animals, neither drug had any effect (-) on non-nociceptive brush-evoked firing, while both drugs were capable of reducing (↓) neuronal firing induced by the noxious mustard oil stimulus. The cold (4°C) stimulus was not tested (n/a) in normal animals, as it elicits no behavioural reflex withdrawal responses in normal animals, only in neuropathic animals. Thus normally, the UPS appears to contribute only to the processing of nociceptive sensory stimuli. In neuropathic animals, proteasome inhibitors are now capable of reducing dorsal horn responses evoked not only by nociceptive mustard oil and newly nociceptive responses to cold stimulation but also responses evoked by non-nociceptive brush stimulation. Thus, in the sensitised state following nerve injury, the UPS is now involved in processing noxious and innocuous sensory stimuli.

The sciatic nerve chronic constriction injury model (CCI) produces sensitisation in the neurons of the spinal dorsal horn and the behavioural correlates of hyperalgesia and allodynia indicative of neuropathic pain [23]. Spinal dorsal horn neurons from neuropathic animals

show elevated responses to brush as well as mustard oil, which is representative of mechanical allodynia, but there is also a *de novo* response to noxious (4°C) cold stimulation, known as 'cold allodynia'. In extracellular recordings from the spinal neurons of neuropathic animals, the same proteasome inhibitors lactacystin and MG-132 could significantly inhibit elevated neuronal responsiveness evoked by noxious stimulation with mustard oil or cold stimulation as well as that evoked by innocuous brush stimulation [21]. This further indicates that ubiquitin is involved in the processing of noxious sensory stimuli, which, following nerve injury, includes not only mustard oil, but now also brush and cold stimulation (***Table 1***).

In agreement with the electrophysiological results, administration of the ubiquitin-proteasome inhibitors MG-132 and epoxomicin into the intrathecal space reversed whole animal behavioural thermal hyperalgesia and mechanical and cold allodynia which develop ipsilateral to nerve injury, but had no effect in naïve animals or on uninjured contralateral paw values [***Figure 2***; 21]. In both behavioural and electrophysiological experiments, proteasome inhibitors exerted an effect within 20 minutes of application. Such rapidity of action suggests that UPS activity is essential for the maintenance of established neuropathic sensitisation.

Biochemical changes were also observed consistent with an involvement of the UPS in the neuropathic pain state. RT-PCR, in situ hybridisation, and Western blot analysis of the rat homologue of ubiquitin C-terminal hydrolase, UCH-L1, (also referred to as PGP 9.5), showed a significant elevation of the expression of mRNA and protein in the spinal cord ipsilateral to nerve injury with a time course paralleling the development of sensitised behavioural reflex withdrawal responses [21]. Thus, there is a clear involvement of the UPS in chronic pain states. But what are the key targets that have implications for spinal cord sensitisation?

POTENTIAL TARGETS OF THE UPS IN NEUROPATHIC PAIN

Protein Kinase A (PKA)

One possible target is protein kinase A (PKA). PKA is composed of regulatory and catalytic subunits, whereby the regulatory subunits normally maintain the kinase in an inactive state. Upon activation, the regulatory subunits of PKA dissociate and there is a translocation to the cell nucleus of the now constitutively active catalytic subunit [24]. Ubiquitin-directed proteolysis is known to degrade the regulatory subunits, leading to persistent kinase activation [13, 24]. PKA is involved in pain transmission, as activation of PKA causes increased dorsal horn neuron responsiveness and leads to hyperalgesia [25, 26, 27] and mice with a knockout of specific regulatory subunits of PKA have reduced responses in the formalin model of inflammation [28]. Furthermore, enzymatic activity of PKA in the spinal cord is enhanced following nerve injury [21]. This increase is blocked by local spinal application of the ubiquitin proteasome inhibitors epoxomicin and MG-132 [21]. Thus, UPS regulation of PKA is important for nociceptive transmission.

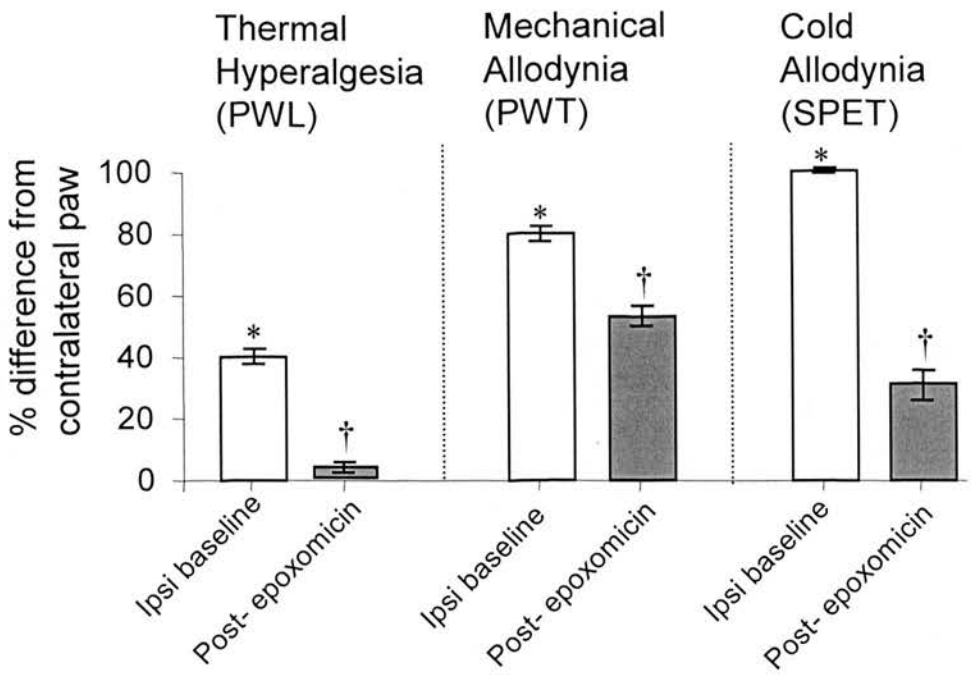

Figure 2: Reversal of neuropathic pain behaviour following epoxomicin treatment.

The bar chart shows mean percentage difference in nerve-injured ipsilateral (ipsi) paw from uninjured contralateral paw, at pre-drug baseline (unshaded columns), and 10-25 minutes following intrathecal administration of the proteasome inhibitor epoxomicin (shaded columns). Data illustrate the percentage change ipsilateral to nerve injury (compared to the contralateral side) in thermal sensitivity (PWL – paw withdrawal latency to noxious thermal stimuli: approximately 40% reduction), mechanical sensitivity (PWT – paw withdrawal threshold to calibrated mechanical stimuli; approximately 80% reduction) and cold allodynia (SPET – Suspended Paw Elevation Test to 4°C water; response seen only on the ipsilateral side). Data are shown as mean ± standard error of the mean. * represents significant ipsilateral-contralateral difference ($p<0.05$, t-test for thermal, Wilcoxon test for mechanical/cold), † represents significant effect of drug ($p<0.05$, One-Way RM ANOVA followed by Dunnett's post-hoc test for thermal, Friedman ANOVA on ranks followed by Dunn's post-hoc test for mechanical/cold). Nerve-injury induced sensitisation is substantially reduced by epoxomicin administration, as indicated by thermal, mechanical and cold sensory tests.

A number of downstream targets of PKA have been identified that are likely to be critical to spinal cord sensitisation and neuropathic pain states and these notably include NMDA and AMPA type glutamate receptors. The C-terminal domains of the NR1 and NR2 subunits of NMDA receptors contain sites of PKA phosphorylation that may increase the degree of Ca^{2+} entry evoked by NMDA receptor stimulation [29, 30, 31, 32]. Electrophysiological responses of spinal neurons to NMDA are known to be facilitated by PKA [12, 25, 26]. NR1 phosphorylation at serine reside 897 (Ser^{897}) is increased in spinal cord following CCI and in spinal dorsal horn spinothalamic tract cells following capsaicin injection [33, 34]. However, it has not yet been established whether there are any PKA regulatory affects on NMDA receptors involving the UPS.

PKA can also phosphorylate serine reside 845 (Ser^{845}) located in the intracellular C-terminal domain of the GluR1 AMPA receptor subunit [35, 36]. Phosphorylation at Ser^{845} increases AMPA receptor responsiveness through a modulation of channel gating and channel open probability without affecting channel conductance [37]. GluR1 subunit phosphorylation at Ser^{845} is known to occur in the superficial (LI/II) spinal dorsal horn in

response to noxious stimulation with capsaicin [38, 39] and following peripheral nerve injury [Garry et al., unpublished observations]. This modulation by PKA is required for the insertion of GluR1-containing receptors into synapses [40]. Correspondingly, the adenylyl cyclase activator forskolin can also elicit recruitment of functional AMPA receptors [16].

NMDA receptors

NMDA receptors are critical to the spinal sensitisation and hyperexcitability that occurs following injury [41, 42, 43]. In the spinal cord, NMDA receptor blockade reduces frequency-dependent potentiation (wind-up) of cells to repeated C-fiber stimulation and following mustard oil application [44, 45]. The NR1 subunit of the NMDA receptor is ubiquitinated by Fbx2, and binding of Fbx2 to the subunit is activity-dependent [46], suggesting that the UPS regulates the NMDA receptor in an activity-dependent manner. Ubiquitination of NR1 in HEK293 cells is blocked by co-expression with the neuronal filament protein, neurofilament-light (NF-L), [47] which has implications for NMDA receptor stability in the PSD [48].

NMDA receptor activation triggers intracellular signalling cascades, which are involved in long-term plasticity. These include two prominent pathways through the nuclear transcription factor cyclic AMP response element binding protein (CREB) and the mitogen-activated ERK-MAP kinase pathways. The CREB pathway activates transcriptional changes, leading to long-term modification of neuronal biochemistry and function. As well as its own profile of transcriptional changes, the MAP kinase pathway causes short-term modification of proteins via phosphorylation, and also activation of CREB [49]. Both pathways are associated with development of chronic pain states. Phosphorylation of p38 MAP kinase and p42/44(ERK1/2) MAP kinase is induced in the ipsilateral dorsal horn of the spinal cord and dorsal root ganglia following peripheral nerve injury (or inflammation) and inhibition of these pathways prevents the behavioural sensitisation seen following nerve injury [50, 51, 52, 53, 54]. Increases in phosphorylation of CREB in the spinal cord are observed following partial sciatic nerve ligation [55] and following chronic constriction injury, with a time-course paralleling development of thermal hyperalgesia [56]. Both pre-treatment with proteasome inhibitors and synaptic blockade augmented NMDA receptor dependent activation of ERK-MAP kinase, and inhibited activation of CREB [57]. CREB is also a prominent target of PKA, which may be activated by a variety of other non-NMDA receptor inputs and which is UPS-regulated (see above). Thus the UPS influences a variety of long-term transcriptional changes through modification of signalling cascades.

AMPA receptors

AMPA receptors are involved in basal nociceptive responses but they are also critical in mediating sensitised sensory responses [58]. Agonists of the AMPA receptor result in sensitisation of behavioural nociceptive responses and increased activity of dorsal horn neurons [59, 60]. AMPA receptor antagonists alter acute nociceptive withdrawal responses and reverse nerve-injury induced pain [9, 58, 61, 62]. Virtually all dorsal horn neurons express GluR2 predominantly in the superficial (LI/II) dorsal horn, where it is known to increase following injury [38, 58, 63]. GluR1 expression is more restricted in the superficial dorsal horn LI/II where it has been localised at primary afferent synapses due to association with markers of unmyelinated afferents [38, 63]. GluR3 and GluR4 are expressed in ventral

and deep dorsal (LIII-VI) horn with weak expression in the superficial dorsal horn and are thus less likely to play a role in pain plasticity [38, 63, 64, 65, 66, 67, 68].

Unlike NMDA receptors, AMPA receptors are rapidly turned over [69, 70], and agonist binding may cause receptor internalisation and breakdown [14, 71]. Insertion and removal of AMPA receptor subunits is now thought to be a major component of synaptic plasticity such that AMPA receptor abundance determines synaptic strength [69, 72, 73]. AMPA receptors undergo rapid endocytosis followed by recycling or degradation in response to ligand binding, NMDA receptor activation; and during long-term depression [14]. Excitatory transmission is depressed following AMPA receptor internalisation [71] and AMPA receptor trafficking from the cytosol to the membrane is thought to be important in hippocampal LTP in culture [73]. It is generally believed that GluR2/3 containing channels are constitutively inserted into the synapse while GluR1/4 containing receptor insertion is inducible (eg. by LTP) [74]. Painful chemical stimulation of the colon or somatosensory nerve injury can cause delivery of GluR1 but not GluR2/3 from cytosol to the plasma membrane in the spinal cord [76, Garry unpublished observations]. However, there is a nerve injury- and AMPA receptor stimulation-induced internalisation of spinal GluR2 that can be prevented by inhibitors of clathrin-coated vesicle endocytosis [58], a mechanism known to regulate AMPA receptor trafficking [75].

AMPA receptor surface expression is subject to regulation by the UPS [15, 16, 77, 79]. There is ubiquitination of GLR-1, the *C Elegans* form of mammalian GluR1, and this has been proposed as a signal for subunit endocytosis [77]. Also, AMPA- (or NMDA-) receptor activation stimulated internalisation of GluR1 and GluR2 in hippocampal dendrites and spinal cord [14, 15, 58, 78];] is prevented by pre-treatment with the proteasome inhibitors MG-132 and lactacystin [15]. These compounds inhibit behavioural reflex sensitisation and suppress dorsal horn neuron hypersensitivity that occurs in response to peripheral nerve injury and noxious stimulation, as mentioned above [21]. This suggests that AMPA receptor trafficking requires UPS-dependent degradation, although no direct ubiquitination of AMPA receptors has been observed [16]. Nevertheless, ubiquitination may occur via a number of intracellular proteins in the PSD that interact with AMPA receptor subunits and are subject to degradation by the UPS[57].

GLUTAMATE RECEPTOR-INTERACTING PROTEINS

While AMPA receptor plasticity is thought to involve changes in the number of receptors at the synapse [72], NMDA receptor plasticity involves a complex of adapter and signalling proteins in the PSD [33, 80]].

The PSD matrix is responsible for the organisation of neurotransmitter receptors, scaffold proteins and signalling enzymes and is dynamically regulated in response to activity. At least some of these changes are mediated by the UPS. Profound changes in the PSD occur in response to prolonged activity, and these are prevented by proteasome inhibition [19]. Indeed, a diverse range of PSD proteins are controlled by UPS-dependent changes.

One such protein is PSD-95, an NMDA receptor-interacting protein that forms intermolecular complexes with signalling and other molecules to regulate receptor function. PSD-95 has a critical role in the development of neuropathic reflex behaviours following nerve injury [33]. Mice with a mutation in PSD-95 that disrupts connections to intracellular

signalling cascades but retains NMDA receptor interacting abilities fail to develop the expected sensitised responses following CCI-induced nerve injury but display normal responses to the formalin model of chronic inflammation [33]. This represented the identification of one of the first proteins underlying a differential processing capacity for different types of chronic pain [81].

PSD-95 can also interact with AMPA receptors to promote their surface expression whereby over-expression of PSD-95 increases both AMPA receptor currents and the number of AMPA receptors at the synapse by inhibition of AMPA receptor internalisation [82, 83, 84, 85, 86, 87]. Stimulation of AMPA receptors leads to a reduction in the number and intensity of PSD-95 puncta in hippocampal dendrites that is sensitive to the UPS inhibitor, MG-132 [82]. This could provide a mechanism for UPS-dependent modulation of AMPA receptor synaptic accumulation. Furthermore, activation of NMDA receptors causes ubiquitination and subsequent degradation of PSD-95 that is prevented in the presence of MG-132 and lactacystin [16]. However, there is some controversy as to the conditions under which ubiquitination of PSD-95 might occur, ie. basally or in response to NMDA receptor stimulation [16, 57, 82]. NMDA receptor stimulation also causes a reduction in the number of synaptic AMPA receptors that is blocked when PSD-95 ubiquitination is prevented [14, 16, 75, 88, 89]; MG-132 can block the loss in synaptic GluR1 subunits that occurs following NMDA receptor activation [15, 16, 78].

PSD-95 interacts indirectly with AMPA receptors through the surface protein stargazin [84, 86]. This trimeric interaction complex is thought to mediate surface expression and synaptic targeting of AMPA receptors [84, 90]. The PDZ binding site of stargazin is regulated by PKA and mutations that mimic this phosphorylation site (T321E and T321D) can downregulate AMPA receptor function [90, 91].

The GluR2 subunit interacts with a variety of intracellular proteins via sequences in its C-terminal tail. GRIP (glutamate receptor -interacting protein) and PICK1 (protein interacting with C kinase 1) have been implicated in AMPA receptor endocytosis [71, 92] and receptor clustering is decreased in cultured spinal neurons with blockade of these interactions [93]. Consistent with this, there is translocation of both GRIP and PICK1 in the spinal cord following AMPA receptor stimulation [58]. Local spinal administration of interfering peptides to block interactions with PICK1 can reverse CCI-induced behavioural reflex sensitisation [58]. The related protein GRIP is also translocated from the membrane in spinal cord following AMPA receptor stimulation [58]. The translocation of both of these intracellular proteins is prevented in the presence inhibitors of the 'clathrin-coated vesicle' pathway/ clathrin mediated endocytosis [58]. However, any involvement of the UPS in this process has not yet been established.

Another GluR2 interacting protein, NSF (N-ethylmaleimide-sensitive fusion protein) is involved in receptor exocytosis [89, 94] and blockade of this interaction in the spinal cord reverses hyperalgesia and allodynia that results from peripheral nerve injury [58]. This interaction is thought to be necessary for normal synaptic expression of AMPA receptors as blocking this interaction in culture results in a rundown of synaptic currents in hippocampal neurons in association with reduced synaptic expression of AMPA receptors [94, 95]. Activity-dependent insertion of AMPA receptors into functionally 'silent' synapses that only express functional NMDA (but not AMPA) receptors may be responsible for the expression

of LTP [96, 97]. Again, it is not known if the UPS is involved in the regulation of AMPA receptor interactions with NSF.

METABOTROPIC GLUTAMATE RECEPTORS

Metabotropic glutamate receptors also play an important role in pain processing, particularly at the level of the spinal cord. Group I mGluRs (mGluR1 and mGluR5) are implicated in the development of sensitised pain states [98], and Group II (mGluR 2 and 3) and Group III (mGluR4 and 7) receptors are implicated in inhibition of pain signalling [99, 100, 101, 102]. Of these, the Group I mGluRs have been shown to undergo ubiquitin-dependent degradation by the protein Siah1A (Seven in absentia homolog 1A), which binds to long splice forms of mGluR1a and mGluR5 [103]. Other mGluRs may also be potential targets for ubiquitination and thereby regulation by the UPS.

THE UPS AND NF-κB SIGNALLING

The UPS regulates gene expression via its interaction with the transcription factor NF-κB [104]. NF-κB normally exists in a dimeric form complexed with the inhibitory regulator, IκB [105]. The IκB complex prevents the nuclear import of NF-κB, holding it in the cytoplasm and thereby inhibiting its transcriptional activity [105]. This interaction is mediated by ankyrin repeats and the ubiquitination and subsequent degradation of IκB by the proteasome is key to NF-κB regulation. The phosphorylation of IκB by IκB kinase (IKK) at two N-terminal serine residues is the event, which precipitates the polyubiquitination of IκB and therefore its degradation [106]. Genes over which NF-κB exerts control and are relevant to neuropathic pain include, proinflammatory enzymes (inducible nitric oxide synthase, cyclooxygenase-2 (COX-2)), cytokines such as tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, chemokines (RANTES, macrophage inflammatory protein, IL-8), adhesion factors (intracellular adhesion molecule, vascular cell adhesion molecule) and proteins involved in controlling apoptosis (Fas, FasL) [107]. Recent evidence has demonstrated that the regulation of NF-κB by the UPS has direct relevance to neuropathic pain as in the CCI model of nerve injury NF-κB activity is increased in rat lumbar DRG ipsilateral to injury [108]. Furthermore, intraneural introduction of competitive inhibitors for NF-κB has been shown to reduce hyperalgesia following spinal nerve ligation [109]. In a recent study we have shown that glial release of cytokines including TNF-α plays a key role in the sensitisation of spinal cord neurons in neuropathic pain [50]. Subcutaneous injection of Complete Freund's adjuvant (CFA), a model of inflammatory pain, has also been shown to cause the immediate activation of spinal NF-κB [110].

In the CFA model, pre-treatment with intrathecally administered inhibitors of NF-κB significantly reduces mechanical allodynia and thermal hyperalgesia [111]. Similarly the I-κB kinase inhibitor, S1627 has been shown to reduce or reverse allodynia and hyperalgesia in both the zymosan-induced model of inflammatory pain and the CCI model, by preventing NF-κB dependant gene expression [107]. These changes in UPS function are likely to have marked effects on NF-κB-mediating signals that bring about central changes in protein expression, which support the development of chronic pain states.

CONCLUSIONS

It is clear that the UPS plays an important part in the sensitization of spinal cord neurons in the neuropathic pain state. Intrathecally administered UPS inhibitors display striking reversal of neuropathic sensitization even when it has become previously established, so may have potential as a novel form of analgesic for this currently intractable pain state. We and others have identified a number of key proteins that are implicated in synaptic plasticity in either spinal cord or forebrain and whose degradation or function is regulated by the UPS. It is unlikely that the role of the UPS in neuropathic pain comprises multiple components with the function of NMDA, AMPA and metabotropic glutamate receptors and major intracellular signalling pathways such as PKA and NF-κB being modulated by UPS activity. Important questions for the future will be unravelling which of these potential contributions are function-limiting in neuropathic pain, whether specific proteins or targeting mechanisms underlie dynamic actions of the UPS in synaptic plasticity and whether agents perhaps targeting such specific actions of the UPS could be developed as useful analgesics. In principle, ubiquitin-proteasome inhibitors would be expected to be beneficial in chronic neuropathic pain states, however, some clinical studies indicate that the first clinically available ubiquitin-proteasome inhibitor, the boronic acid derivative, bortezomib actually caused peripheral neuropathy de novo or perhaps exacerbated a pre-existing peripheral neuropathy, such as Charcot-Marie-Tooth disease [112]. It is not clear if this neuropathy develops because the agent is blocking the UPS or is a side effect of this particular drug, as is found, for example with some other chemotherapeutic drugs such as the vinca alkaloid, vincristine [113, 114]. Only trials with other ubiquitin-proteasome inhibitors of different chemical structures, but the same effect will reveal this.

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